PHD

Studies on the nicotinic acetylcholine receptor of the locust

Macallan, David Robert Edward

Award date:
1988

Awarding institution:
University of Bath

STUDIES ON THE NICOTINIC ACETYLCHOLINE RECEPTOR OF THE LOCUST

Submitted by **DAVID ROBERT EDWARD MACALLAN**

for the degree of Ph.D.

of the University of Bath

1988

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

I am grateful to the Science and Engineering Research Council for financial support throughout the course of this project.

My thanks are due to many people, past and present, in the Biochemistry department for their help during this project and in particular Dr Sue Wonnacott for stimulating discussion, Mr Richard Kinsman and Miss Sue O'Neil for help with the peptide work, and Miss Colette O'Sullivan and Miss Sue Walsh for the antibodies.

I would also like to thank Mr John Marshall for help with the oocyte work and Miss Susan White for typing this thesis. Particular thanks go to my supervisor Dr George Lunt for his continued encouragement and help throughout this work.

Finally, I would like to thank Astrid for her great moral support.

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SUMMARY

SUMMARY

The binding of (^{125}I) α bungarotoxin and (^{3}H)(-)nicotine to a membrane fraction of the supraoesophageal ganglion from the locust Schistocerca gregaria was compared. For (^{125}I) α bungarotoxin binding a Kd and Bmax of 0.8 nM and 1.2 pmol/mg protein were calculated whereas the Kd and Bmax for (^{3}H)(-)nicotine binding were calculated to be 130 nM and 4 pmol/mg protein. A pharmacological profile for these two binding sites was made. In the case of the (^{125}I) α bungarotoxin binding site, distinct cholinergic pharmacology was evident consistent with this site being part of an insect neuronal nicotinic acetylcholine receptor. Dihydro β erythroidine, a classical nicotinic antagonist, and two naturally occuring toxins, methyllycaconitine and (+)anatoxin-a, were found to be particularly potent at this site. Furthermore, the binding of (^{125}I) α bungarotoxin to this tissue shares pharmacological specificity with both vertebrate neuronal and peripheral nicotinic acetylcholine receptors. In contrast the (^{3}H)(-)nicotine binding site appeared to be non-cholinergic in nature and the significance of this binding site is unclear. This (^{3}H)(-)nicotine binding site did not bind the specific neuronal high affinity nicotine binding site ligand (^{3}H)methylcarbamylcholine.

The αbungarotoxin binding protein from locust ganglia was purified by affinity chromotography and on silver stained denaturing polyacrylamide electrophoresis gel a predominant band at Mr 49,000 was consistently present in all the preparations made. This corresponds exactly to the Mr of a polypeptide in the ganglionic membrane fraction that is labelled by the nicotinic affinity ligand (^{3}H)MBTA and it is likely that these two polypeptides are identical.

Poly A+ mRNA extracted from locust ganglia was shown to direct the synthesis of the α bungarotoxin binding component in Xenopus oocytes.

An antiserum was raised against a synthetic peptide, the sequence of which was derived from a clone isolated from a genomic DNA library of Schistocerca gregaria and corresponds to the 'cysteine loop' region present in all ion channel receptor protein subunits. This antiserum was shown to react with polypeptides of Mr 49,000 and 60,000 in Western blots of the ganglionic membrane fraction. Moreover, the antiserum reacted with the purified α bungarotoxin binding protein in Western blots. An antiserum raised against the purified α bungarotoxin binding component recognised the synthetic peptide in a dot immunobinding assay. It is concluded that this genomic clone encodes a protein that is similar to that purified by αbungarotoxin affinity chromatography.

ABBREVIATIONS

ACh	:	Acetylcholine
AChE	:	Acetylcholine esterase
AChR	:	Acetylcholine receptor
ANS	:	Autonomic nervous system
BACh	:	Bromoacetylcholine
Bmax	:	Maximum number of binding sites
BSA	:	Bovine Serum albumin
cAMP	:	adenosine-3',5'-monophosphate
ChAT	:	Choline acetyltransferase
CNBr	:	Cyanogen bromide
CNS	:	Central nervous system
DCM	:	Dichloromethane
DHβE	:	Dihydro β erythroidine
DMF	:	N,N-dimethyl formide
DMPP	:	1,1-Dimethyl-4-piperazinium
EMS	:	Ethylmethylsulphide
GABA	:	δ-Aminobutyric acid
HPLC	:	High performance liquid chromatography
IC_{50}	:	Concentration of inhibitor giving 50% displacement
Kd	:	Dissociation constant
Ki	:	Inhibition constant
Mab	:	Monoclonal antibody
mAChR	:	Muscarinic acetylcholine receptor

MBTA	:	4-(N-maleimido)-benzoyltrimethylammonium iodide
MCC	:	Methylcarbamyl choline
MLA	:	Methyllycaconitine
Mr	:	Relative molecular weight
nAChR	:	Nicotinic acetylcholine receptor
NGS	:	Normal goat serum
PBS	:	Phosphate buffered saline
PEI	:	Polyethyleneimine
PI	:	Phosphatidyl inositol
PMSF	:	Phenylmethane sulphonyl fluoride
SD	:	Standard deviation
SDS	:	Sodium dodecyl sulphate
SDS-PAGE	:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAA	:	Tertiary amyl alcohol
TEA	:	Tetra ethyl ammonium
TFA	:	Trifluoro acetic acid

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Insects and the Pesticide Industry

Of the 1,200,000 identified species in the animal kingdom, 97% are invertebrates. Moreover, 78% of the total identified species belong to the class Insecta. However, there is still a large number of insect species yet to be identified and the most accurate estimates suggest that there may be between four and five million individual species of insect on the earth's surface. Insects occur practically everywhere and have inhabited the earth for nearly 300 million years. As a result they have adapted to live in almost every kind of habitat available and have evolved to overcome the various problems of food supply and protection against predetors.

The insects have always presented great problems to man. Not only are they responsible for transmission of many diseases but they are responsible for a great loss of agricultural produce. In fact it has been estimated that some 14% of the world's agricultural production is lost by insect attack each year and this figure does not include losses in pastoral farming or the loss of animal stock due to insect transmitted diseases (Cramer, 1967). Not all insects can be classified as pests and man has gained many benefits from the presence of insects, such as the pollination of crops and silk production.

What then has man's approach to the problem of insect control been? In the 1930's there was widespread use of lead arsenate which was an effective insecticide but had the disadvantage that it was also poisonous to man. Nicotine has long been known to be a potent insecticide (Friedlander, 1976), acting as a neuropoison, but again is just as poisonous to man and is unstable in air. Just before the second world war a new insecticide was in use, which was extremely toxic to insects but harmless to man. This compound was pyrethrum, an extract of the daisy Pyrethrum cinerariaefolium. However, the main supplies of pyrethrum came from Japan and Kenya and therefore these sources quickly dried up with the arrival of the war. There was a desperate need for a new widely acting insecticide. The discovery of para-dichloro diphenyl trichloroethane (DDT) filled this gap and marked the start of industrial insecticide development, mainly using the methods of random synthesis and screening. At present there are four main classes of insecticide in use : the organochlorides; the organophosphates; the carbamates; and the pyrethroids. However, the exact target at the molecular level for the majority of these insecticides is still largely unknown.

Many lessons have been learnt from the widescale use of insecticides. Two main factors responsible for the unforeseen problems encountered were the lack of pesticide

specificity and the problem of resistance. The first of these has been noted in many situations where the parasites and predetors that normally hold the insect in check have proved to be more susceptible to the insecticides than the pest itself. The problem of resistance has been demonstrated most effectively in the control of the cotton weevil which in 1967 was resistant to all known insecticides of that time (Mathews, 1983).

There is now a major market for new, highly specific insecticides and the insecticide companies are now starting to adopt more rational design approaches in addition to their synthesis and screen programmes. Therefore, further research into basic neurobiology of insects is required before potential targets for insecticides can be identified. At present, our knowledge of the insect nervous system is rather limited when compared to that of vertebrate species. However, considerable progress is being made in our understanding of the cholinergic system in insects and the work detailed in this project is focused on the nicotinic acetylcholine receptor (nAChR) of the desert locust.

The full taxonomic classification of the desert locust, Schistocerca gregaria, is shown in figure 1. Locusts belong to the order Orthoptera which have the following characteristics : the mouthparts are simple and suited for

biting; the fore-wing is harder than the membranous hind-wing and in flight is not flapped but held out sideways like an aeroplane's wing; the hind legs are modified for jumping; there are usually organs for sound production and reception.

1.2 The Insect Nervous System

The insect central nervous system (CNS) is made up of a number of paired ganglia, lying along the length of the body, which are connected to each other longitudinally to form the ventral nerve cord (figure 2). In the embryonic stage of the insect, each segment of the body contains its own paired ganglion. Some of these ganglia fuse before the insect emerges from the egg and the degree of fusion depends on the individual species. Minimal degree of fusion is seen in species of the order Dictyopterus whereas the maximum degree of fusion is seen in species of the genus Musca. In the adult locust, there is a fused neural mass in the most anterior part of the head, the supraoesophageal ganglion, which is made up of one or more segmental ganglia along with the primitive pre-segmental archecerebrum. This ganglion is the centre for receiving all stimuli from the sense organs in the head as well as receiving excitatory impulses from the ventral nerve cord. It is therefore the major centre of association and to some extent controls the activity of the rest of the nervous system. It may also be involved in the process of

learning and the regulation of long-term organised behaviour patterns. The supraoesophageal ganglion is composed of three main areas, the protocerebrum, the deutocerebrum and the tritocerebrum (figure 3). The protocerebrum is continuous laterally with the optic lobes and is the most complex part of the brain. The deutocerebrum is the point of origin of the antennal lobes. The circumoesophageal commissure connecting the supraoesophageal ganglion to the suboesophageal ganglion originates from the tritocerebrum and the tritocerebral lobes are connected by the frontal commissure. The supraoesophageal ganglion is more complex than ganglia of the ventral nerve cord containing many neuropile regions.

1.3 The 'neurone doctrine'

In the middle of the 18th century there was much support for the theory that the brain was not composed of cells, but was a continuous network of vessels resembling the blood circulatory system. Long extensions originating from cell bodies were clearly visible in the observations of Deiter published in 1865. However, there was no evidence to suggest that these extensions had terminal ends completely surrounded by the cell membrane. In 1885 the first piece of evidence in support of individual nerve cells was published by Golgi using a method of staining which identified only a few of the nerve cells present. There was little interest shown in this until 1888 when

Ramon y Cajal modified Golgi's original staining method and identified what appeared to be single cells in many brain regions. Acceptance of this proposal soon followed and in 1891 Waldeyer published a review of evidence supporting the existence of nerve cells calling them neurones and the nervous system became known as the "neurone doctrine". Final proof of individual nerve cells came from the use of the electron microscope in the 1950's, which showed that the nerve cell membrane, like the membrane of other cells, was continuous around each nerve cell.

1.4 Cellular Structure of Insect Ganglia

All insect ganglia have a similar basic structure as shown in figure 4. There is an outer layer, neural lamella, surrounding the ganglion beneath which lies a layer of cells called the perineurium. The nerve cell bodies (perikarya) are found below the perineurium and are enveloped by one or more glial cells. In the centre of the ganglion is the neuropile region where all the synaptic connections are found. There are no perikarya and little or no glial cells present in the neuropile region. In contrast to the vertebrate nervous system, there are no synapses onto the cell bodies of insect neurones. Tracheoles are present in the ganglia although there is no vascular system and metabolites have to diffuse from the haemolymph through the various layers of nervous tissue.

1.5 Synaptic Transmission

It was Ramon y Cajal who proposed the idea that nerve signals pass along dendrites and axons and that transmission between nerve cells occur only at points where the axons and dendrites come into contact. This was later confirmed by the electrophysiological studies of Sherrington (1925), who suggested that the point of contact between one nerve cell and another, or between a nerve and a muscle cell be called a synapse, a word meaning to clasp, connect or join. In 1904 Eliot's experiments suggested that an epinephrine-like substance was released from nerve terminals onto effector cells in the adrenal gland in response to electrical stimulation of the nerves. More definite evidence in support of chemical transmission came from the experiments of Loewi (1921), who demonstrated that chronic stimulation of the vagus nerve resulted in the release of a chemical (acetylcholine) into the bathing medium and that this medium could then mimic vagal stimulation. Conclusive proof of synaptic transmission came from the identification of nerve terminals and synaptic vesicles, again using the electron microscope.

1.6 Cholinergic Transmission

1.6.1 The history of acetylcholine

Acetylcholine (ACh) was the first neurotransmitter to be identified and the study of cholinergic transmission can

be traced back to the middle of the 19th century, when Bernard carried out some experiments in which he demonstrated that skeletal muscle contraction in response to nerve stimulation could be blocked by the application of curare. This was followed by Langley (1905) who demonstrated that the application of nicotine to skeletal muscle resulted in contraction which could be prevented by curare, and he was the first to propose the presence of receptor molecules which he called "receptive substances". In 1914, Dale identified two types of cholinergic activity, muscarinic and nicotinic, in the nervous system based on the effects of nicotine and muscarine. Dale then proceeded to provide evidence that ACh acts as a neurotransmitter in autonomic ganglia and at the neuromuscular junction.

1.6.2 The cholinergic synapse

Considerable knowledge of the structure and events at the cholinergic synapse is now known, and is summarised in Figure 5. ACh is synthesised from AcetylCoA and choline by the enzyme choline acetyltransferase (ChAT). Newly synthesised ACh is stored in synaptic vesicles. An action potential arriving at the synapse leads to the depolarisation of the nerve terminal. This in turn allows an influx of Ca^{++} ions which are required for the release of ACh from the synaptic vesicles into the synaptic cleft. The released ACh then crosses the cleft and binds

reversibly to a receptor protein situated in the post-synaptic membrane. The result of this binding is a cellular response. In the case of nAChR's, the response is an increase in conductance of Na^+ ions. Under non-physiological conditions, other cations such as K^+ and Ca^{++} will also pass through the nAChR channel. A variety of responses are linked to muscarinic acetylcholine receptors (mAChR's) including an increase in the concentration of cGMP, a decrease in cAMP and the breakdown of polyphosphoinositides. These three responses are thought to be responsible for changes in K^+ ion permeability and may occur sequentially or independently. Released ACh may also act at presynaptic autoreceptors. The nicotinic autoreceptor is thought to increase ACh release (Moss and Wonnacott, 1985) whereas the muscarinic autoreceptor may have a negative feedback effect terminating synaptic release of ACh (Polak, 1971; Nordstrom and Bartfai, 1980). The ACh in the synaptic cleft is prevented from accumulating by the action of acetylcholine esterase (AChE), a membrane bound enzyme which hydrolyses ACh to acetate and choline. Free choline is then taken back into the presynaptic terminal by a high affinity choline carrier and can be reincorporated into ACh.

1.6.3 The insect cholinergic synapse

Most of the cholinergic elements described in the previous section have been identified in insect nervous tissue. Very high activities of ChAT have been detected in the

nervous system of the moth Manduca sexta (Prescott et. al., 1977), antibodies have been used to localise this enzyme in the CNS of the fruitfly (Drosophila melanogaster) (Buchner et. al., 1986) and it has been purified from ganglia of the locust Schistocerca gregaria (Lutz et. al., 1988). ACh has been localised to the neuropile region of insect ganglia (Colhoun, 1963). Moreover, synaptosomes prepared from Locusta migratoria ganglia (Breer, 1981b) have been shown to contain high quantities of ACh and synaptic vesicles have been isolated which contain ACh (Takeno et. al., 1981). Synaptic release of ACh in response to depolarising agents in a Ca^{++} dependent process has been demonstrated (Breer and Knipper, 1984) and choline has been shown to be taken up by a high affinity choline carrier into nerve endings (Breer, 1982). Post-synaptic depolarisations resulting from changes in membrane permeability in response to released ACh have been detected (Sattelle, 1980; Sattelle et. al., 1980, 1983). Additionally, these responses can be mimicked by locally applied acetylcholine (Callec, 1974; Callec et. al., 1982) and nicotinic agonists (Sattelle, 1978) but are prevented by nicotinic antagonists (Sattelle, 1978; Sattelle et. al., 1983). Muscarinic cholinergic responses to released ACh are not well understood in insect tissue but are thought to be linked to changes in PI turnover (Trimmer and Berridge, 1985; Duggan and Lunt, 1986) and changes in the concentration of cAMP (Duggan and Lunt, 1986).

1.7 Vertebrate Nicotinic Acetylcholine Receptors

Nicotinic AChR's have been identified in both peripheral tissue (neuromuscular junction and electroplaque) and nueronal tissue (CNS and autonomic nervous system (ANS)). The peripheral nAChR is the most extensively characterised of all the neurotransmitter receptor proteins known. This is attributed to two main factors. Firstly, the use of the electric organ (electroplaque) from the electric fish Torpedo and the electric eel Electrophorus. This specialised tissue is a very rich source of nAChR's and most of the early work on this protein was performed using this tissue. The second important factor was the availability of the snake venom αneurotoxins from the Elapid snakes (Bungarus multicinctus and various species of Naja) and in particular αbungarotoxin which is an extremely potent antagonist, binding essentially irreversibly to the nAChR. In contrast our knowledge of neuronal nAChR's is far less advanced and this in part may be due to the unsuitability of αbungarotoxin as a probe for these receptors (see section 1.10.1) and the low abundance of these receptors in neuronal tissues.

1.8 The Torpedo nAChR

The characteristics of the Torpedo nAChR have been reviewed many times (Conti-Tronconi and Raftery, 1982; Changeux et. al., 1984; Dolly and Barnard, 1984; McCarthy et. al., 1986). The protein has been affinity purified

from detergent extracts using immobilised α neurotoxins (Raftery, 1973; Karlsson et. al., 1972) and has a relative molecular weight (Mr) between 250,000 and 300,000 depending on the method of determination (Hucho, 1986). It consists of four types of subunit assigned α, β, γ and δ with Mr 40,000, 50,000, 60,000 and 65,000 respectively, which form a pentameric structure in the ratio $\alpha_2\beta\gamma\delta$. Small angle X-ray diffraction studies have determined the overall shape of the pentameric protein in the membrane, shown in Figure 6. The nAChR from Torpedo is found predominantly as dimers in the membrane (Conti-Tronconi and Raftery, 1982), linked by a disulphide bridge between the δ subunits. However the functional significance of this structure is unclear and the dimeric form is not observed in tissues from any other species (Hucho, 1986). A protein of Mr 43,000 is associated with the receptor and is thought to be a structural link between the nAChR and the cytoskeleton (Porter and Froehner, 1985; Walker et. al., 1984).

1.8.1 Primary structure of the subunits

The full coding sequences for each of the subunits of the Torpedo californica nAChR were determined from cDNA clones isolated with oligonucleotide probes (Noda et. al., 1982, 1983b, 1983c; Claudio et. al., 1983) corresponding to the N-terminal sequence of each subunit which had been previously determined by protein sequencing (Devillers-Thiery et. al., 1979; Raftery et. al., 1980). The

α subunit sequence of Torpedo marmorata has also been determined in this way and shows extensive homology to the Torpedo californica α subunit (Devillers-Thiery et. al., 1983). Comparing the sequences of the subunits there is about 40% homology with α being more homologous to β than to γ or δ and γ being more homologus to δ than to α or β. This suggests that the four subunits have diverged from two and originally one ancestral gene (Raftery et. al., 1980).

1.8.2 Functional expression of cloned nAChR's

Functional expression of mRNA's for the four subunits derived from the cDNA clones has been achieved in the oocyte expression system (Mishina et. al., 1984) but not in cell free translation systems. This can be attributed to the requirement for cellular functions and the presence of the cell membrane for correct post-translational modifications such as cleavage of leader sequences, glycosylation, methylation and acylation (Hucho, 1986). Site directed mutagenesis has established that the asparagine at position 141 (α subunit sequence) is the site of glycosylation and is important for receptor function (Mishina et. al., 1985).

1.8.3 Biochemical evidence for an integral ion channel

Evidence in support of the presence of an integral ion channel came from $^{22}Na^{+}$ flux and patch clamp experiments

on reconstituted purified nAChR's (Schiebler and Hucho, 1978; Epstein and Racker, 1978; Schindler and Quast, 1980) and oocyte expressed nAChR's (Mishina et. al., 1984). Hybrid expression studies using Torpedo α, β, β, and γ subunits and the δ subunit of calf muscle receptor has indicated that the δ subunit may be involved in the gating of the ion channel (Sakemann et. al., 1985).

1.8.4 Tertiary structure of the nAChR

The exact tertiary structure of the individual subunits of the nAChR is still unknown since crystalisation of the protein followed by X-ray analysis has not yet been possible. Models of the tertiary structure of predicted secondary structures have been proposed based on the results from biochemical studies, electron microscopy and hydrophobicity analysis of the subunit sequences (Guy and Hucho, 1987) and these are shown in Figure 7. Four transmembrane regions (M1, M2, M3 and M4) were identified from the amino acid sequence and both the N-terminal and the C-terminal were initially thought to be extracellular which led to the proposal of model 1 in Figure 7 (Noda et. al., 1982; Devillers-Thiery et. al., 1983; Claudio et. al., 1983). However monoclonal antibodies have located the C-terminal region to the cytoplasmic side of the membrane (Lindstrom et. al., 1986) and since this requires five crossings of the membrane, a fifth transmembrane region (MA) was proposed, consisting of alternating polar

and hydrophobic residues, which was thought to form the ion channel, leading to the proposal of model 2 in Figure 7 (Guy, 1984; Finer-Moore and Stroud, 1984). Mutagenesis experiments have implicated that the region connecting MA to M4 may be associated with the agonist binding site and therefore on the extracellular side thereby lending support to model 2 (Mishina et. al., 1985). However, the regions MA and M4, and the portion of the polypeptide chain linking these segments, were shown to be on the cytoplasmic side of the membrane using monoclonal antibodies (Ratnam et. al., 1986a, 1986b) and this has led to the proposal of a third model of the tertiary structure (model 3 Figure 7) where the MA and M4 regions are located intracellularly (Ratnam et. al., 1986a). In contrast, the intracellular presence of the MA and M4 regions has also been explained by a non-functional conformation of model 2 where the regions MA and M4 only span the membrane when the five subunits have aggregated (Finer-Moore and Stroud, 1984). Since all four types of subunit are highly homologous to each other it is believed that they will all have the same tertiary structure. In model 3 the transmembrane region M6 is proposed to form the ion channel whereas in model 2 it is the MA region that is thought to form the ion channel. However, recent experiments using triphenylmethylphosphonium (TPMP), a channel blocker, suggest that M2 is involved in the formation of the ion channel (Giraudat et. al., 1986;

Hucho et. al., 1986) perhaps in conjunction with MA (Guy and Hucho, 1987).

1.8.5 Localisation of the ligand binding site

The α subunit of the nAChR has been shown to contain the binding site for nicotinic agonists and competitive antagonists (for review see Hucho, 1986). However, one of the α subunits in the pentamer has a higher affinity for d-tubocurarine than the other and the kinetics of α bungarotoxin binding to the two α subunits are different. The affinity ligands 4-(N-maleimido) benzyltrimethylammonium iodide (MBTA) and bromoacetylcholine (BACh) also bind preferentially to one of the α subunits. Both of these affinity ligands label the sulphydryl groups in the ligand binding site and it has been demonstrated that MBTA labels the cysteine residues at positions 192 and 193 which are only present in the α subunit (Kao et. al., 1984). Using synthetic peptides, the region 190-193 has been implicated as being involved in the binding of α bungarotoxin (Lindstrom et. al., 1986) although there are reports suggesting that synthetic peptides corresponding to various other regions also bind α bungarotoxin (Atassi et. al., 1987). It seems likely that the ligand binding site is composed from various regions of the primary structure brought into close vicinity by the three dimensional structure of the protein, but includes the important adjacent cysteine residues at positions 192 and 193. The true three

dimensional structure of the cholinergic binding site will probably be revealed by X-ray crystalography of the protein.

1.9 The Muscle nAChR

The vertebrate muscle nAChR has been characterised from many species and is similar to the electroplaque receptor with respect to structure and pharmacology. This is expected since electroplaque cells are derived from embryonic striated muscle cells. The amino acid sequence of each of the subunits of the receptor has been determined for chick muscle (Barnard et. al., 1986), mouse muscle (Heinemann et. al., 1986) and calf muscle (Noda et. al., 1983a; Tanake et. al., 1984; Takai et. al., 1984; Kubo et. al., 1985; Takai et. al., 1985). It is clear that there is strong homology between these muscle nAChR subunits and the corresponding subunits of the electroplaque receptor (Kubo et. al., 1985). However, slight differences are observed for muscle receptors when compared to the Torpedo receptor. In the chick muscle receptor the β subunit is thought to have a higher Mr than the γ subunit (Barnard et al., 1986). Also, an additional subunit has been identified in calf muscle, termed ε, which may replace the γ subunit during muscle development (Mishina et.al., 1986).

1.10 Vertebrate Neuronal nAChR's

Both the CNS and ANS of vertebrates are known to contain nAChR's (Martin, 1986; Wonnacott, 1987). These receptors have been identified primarily by intracellular recording of depolarisations evoked by iontophoretically applied ACh and nicotinic agonists (Curtis and Crawford, 1969; Oswald and Freeman, 1981; Schuetze and Role, 1986). Ganglionic nAChR's can be blocked by hexamethonium, a C6 nicotinic antagonist, but not by decamethonium, a C10 nicotinic antagonist potent at the muscle receptor. There may be both C6 and C10 type nAChR's present in vertebrate CNS.

1.10.1 αBungarotoxin as a probe of neuronal nAChR's

There are conflicting reports on the ability of α bungarotoxin to block synaptic transmission in both the CNS and ANS. In higher vertebrate species, αbungarotoxin has been shown to be ineffective in blocking central (Duggan et. al., 1976; Brown et. al., 1983) and ganglionic (Brown and Fumigalli, 1977; Carbonetto et. al., 1978; Ravdin and Berg, 1979; Kouvelas et. al., 1978; Kato and Narahashi, 1982) synaptic nAChR's. In contrast, α bungarotoxin has been shown to block transmission in the retino-tectal synapses of the toad (Freeman, 1977; Freeman et. al., 1980) and goldfish (Freeman et. al., 1980; Schmidt and Freeman, 1980) and this receptor is thought to be located presynaptically (Henley et. al., 1986; Langdon and Freeman, 1987). However, no inhibition

of retino-tectal synaptic transmission by αbungarotoxin in rat brain was observed (Schmidt and Freeman, 1980). Inhibition of synaptic transmission by αbungarotoxin has also been demonstrated in frog sympathetic neurones (Marshall, 1981), the superchiasmatic nucleus of the rat hypothalamus (Zatz and Brownstein, 1981) and the rat inferior colliculus (Farley et. al., 1983). Finally, extrajunctional nAChR's have been shown to be present in the rat superior cervical ganglion which can be blocked by α bungarotoxin (Dun and Karczmar, 1980) although the functional significance of extrajunctional receptors is unclear.

1.10.2 Biochemical identification of neuronal nAChR's

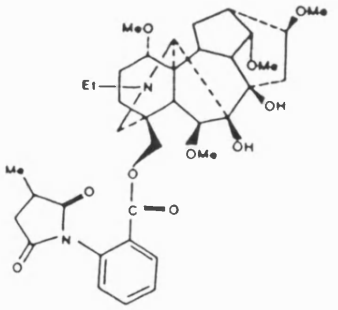
Radiolabelled αbungarotoxin, (-)nicotine and ACh have all been used to identify neuronal nAChR's (for review see Wonnacott, 1987). αBungarotoxin binding sites showing cholinergic pharmacology have been identified in both the CNS and ANS (Morley et. al., 1979; Morley and Kemp, 1981; Oswald and Freeman, 1981; Schuetze and Role, 1986) although the functional significance of these sites is unclear. These sites are found mainly extrasynaptically and some are found on cells lacking any known cholinergic innervation. Nicotine and ACh appear to bind to identical sites whose distribution is different from that of α bungarotoxin (Clarke et. al., 1985, 1986). High affinity nicotine binding sites in the CNS have been

extensively characterised and show cholinergic pharmacology but are insensitive to αbungarotoxin (Wonnacott, 1987). Therefore, there appear to be at least two distinct types of nAChR in vertebrate brain, based on the ability to bind αbungarotoxin. More recently, a new radioligand (^{3}H)methylcarbamylcholine (MCC) has been shown to be a specific ligand for the neuronal high affinity nicotine binding site (Abood and Grassi, 1986; Boksa and Quirion, 1987) and these sites have been localised by autoradiography with this ligand (Yamada et. al., 1987).

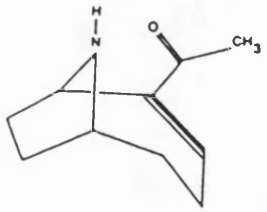
1.10.3 Other naturally occuring toxins as probes of neuronal nAChR's

Another toxin sometimes present in commercially available α bungarotoxin samples has been isolated (kappa bungarotoxin, also called toxin 3.1 and toxin F) which is able to block synaptic transmission in the chick ciliary ganglion (Chiapinelli, 1985). This toxin has a Mr of 6,500 on denaturing gel electrophoresis and shows homology of about 47% with αbungarotoxin. Kappa bungarotoxin binds to two sites in the chick ciliary ganglia one of which is the αbungarotoxin binding site and the other may be a high affinity nicotine binding site. More recently, (+)anatoxin-a, a product of the fresh water alga Anabaena flos-aquae, has been shown to be an extremely potent inhibitor of (^{3}H)(-)nicotine binding in rat brain with a lower affinity for the rat brain αbungarotoxin binding

site (MacAllan et. al., 1988) and was shown to be a potent agonist of a nAChR in cultures of rat hippocampal cells (Aracava et. al., 1987). This toxin has previously been demonstrated to be a potent agonist at the vertebrate neuromuscular junction closely resembling acetylcholine in its channel properties (Albuquerque and Spivak, 1984; Swanson et. al., 1986). Methyllycaconitine (MLA), a component found in the seeds of Delphinium browni, is a potent insecticide (Jennings et. al., 1986) but is only moderately effective as an antagonist at the mammalian neuromuscular junction (Nambi-Aiyar et. al., 1979). This toxin has also been shown to be an effective inhibitor of (^{125}I)αbungarotoxin binding in rat brain (MacAllan et. al., 1988).



METHYLLYCACONITINE



ANATOXIN-A

1.10.4 Purification and characterisation of neuronal nAChR's

Using the techniques developed for the electroplaque nAChR, αbungarotoxin binding sites have been purified from neuronal tissue of rat (Wonnacott et. al., 1982; Kemp et. al., 1985), mouse (Seto et. al., 1981), goldfish

(Oswald and Freeman, 1979), chick optic lobe and chick brain (Norman et. al., 1982; Conti-Tronconi et. al., 1985). These proteins have been shown to be labelled by the affinity ligands MBTA and BACh (Lukas and Bennett, 1980). The α bungarotoxin binding component from chick brain was initially found to have only one subunit of Mr 54,000 (Norman et. al., 1982) but was subsequently shown to consist of three subunits of Mr 48,000, 56,000 and 69,000 (Conti-Tronconi et. al., 1985), with the 56,000 subunit being labelled with BACh. Additionally, monoclonal antibodies directed against the chick muscle nAChR were shown to cross react with this chick brain α bungarotoxin binding component (Mehraban et. al., 1984). The purified rat brain α bungarotoxin binding component was found to consist of three subunits of Mr 49,000, 53,500 and 55,000 and it was the 55,000 subunit that was labelled with MBTA (Kemp et. al., 1985). This α bungarotoxin binding component of rat brain has been separated from the high affinity nicotine binding site using an α bungarotoxin affinity column (Wonnacott, 1986) and this provides further evidence that these two binding sites are separate entities.

Rapid progress is now being made on the purification of the high affinity nicotine binding site which is insensitive to α bungarotoxin. Using a monoclonal antibody (Mab 35) against the Torpedo nAChR, a protein

complex consisting of two types of subunit with Mr 49,000 and 59,000 has been purified from chick brain (Whiting and Lindstrom, 1986a). This protein was labelled with BACh which could be blocked by carbachol but not by α bungarotoxin (Whiting and Lindstrom, 1986a). The same protein could bind (^{3}H)(-)nicotine with high affinity which could be blocked by various nicotinic cholinergic ligands but not by αbungarotoxin (Whiting and Lindstrom, 1986b) and it was the 59,000 subunit of this protein complex that was labelled with MBTA (Whiting and Lindstrom, 1987a). A second nAChR has been identified in chick brain, using a monoclonal antibody (Mab 270) against the previously purified chick brain nAChR, which has been shown to consist of a protein complex of subunits with Mr 49,000 and 75,000 (Whiting et. al., 1987a). This antibody has further been used to purify the rat brain high affinity nicotine binding site (Whiting and Lindstrom, 1987b) which consists of two subunits with Mr 51,000 and 79,000. Mab 270 has also been used to localise nAChR's in rat and mouse CNS and the pattern of distribution was found to be similar to the distribution of (^{3}H)(-)nicotine binding sites in these tissues (Swanson et. al., 1987). Functional nAChR's have been identified on PC12 cells using Mab 270 (Whiting et. al., 1987c) which are distinct from the αbungarotoxin binding sites also present on these cells. More recently, monoclonal antibodies against the rat brain nAChR have been used to study bovine and

human neuronal nAChR's (Whiting and Lindstrom, 1988). The immunoaffinity purified bovine nAChR consists of two subunits with Mr 50,600 and 74,400 and is therefore very similar to rat and chick brain nAChR's. A nAChR has also been purified from rat brain using an affinity column made with (-)-6-hydroxymethyl-nicotine and this purified protein has been characterised with idiotypic and anti-idiotypic antibodies to nicotine (Abood et. al., 1987). This protein complex consists of two subunit types with Mr 57,000 and 62,000 and is therefore different from the protein purified by Whiting and Lindstrom (1987b).

1.10.5 Molecular cloning of neuronal nAChR's

Using cDNA probes of the muscle nAChR subunits, cDNA's coding for subunits of neuronal nAChR's have been isolated. Three types of α subunit have been identified in rat neuronal tissue, designated $\alpha 2$, $\alpha 3$ and $\alpha 4$, but only one β type subunit, $\beta 2$, has been identified in this tissue. The rat brain $\alpha 2$ gene was isolated from a rat brain cDNA library using the rat $\alpha 4$ clone as a probe (Wada et. al., 1988). The rat brain $\alpha 3$ gene was isolated from a cDNA library of PC12 cells using a mouse muscle α subunit probe (Boulter et. al., 1986). The rat brain $\alpha 4$ clone was isolated from cDNA libraries of rat hypothalamus and hippocampus using a mouse muscle αsubunit probe and the $\alpha 3$ probe (Goldman et. al., 1987). The rat brain $\beta 2$ clone was isolated from a cDNA library

of PC12 cells (Boulter et. al., 1987). The three rat brain α clones are thought to code for agonist binding subunits whereas the rat brain $\beta 2$ clone is thought to be a structural subunit. It now seems likely that the rat brain $\alpha 4$ clone encodes the rat brain 79,000 Mr subunit (Whiting et. al., 1987b). Functional expression of $\beta 2$ with $\alpha 2$ (Wada et. al., 1988) and $\beta 2$ with either $\alpha 3$ or $\alpha 4$ (Boulter et. al., 1987) has been demonstrated in the oocyte expression system. These expressed receptors are activated by ACh and nicotine but are insensitive to α bungarotoxin. The receptors expressed by $\alpha 3$ and $\alpha 4$ in conjunction with $\beta 2$ were shown to be blocked by toxin 3.1 whereas the receptor expressed by $\alpha 2$ and $\beta 2$ was insensitive to this toxin. Additionally, expression of $\alpha 4$ on its own also gave weak nicotinic response. More recently genes corresponding to $\alpha 2$, $\alpha 3$, $\alpha 4$ and $\beta 2$ of rat brain have been isolated from the chick genome ($\alpha 2$, $\alpha 3$, $\alpha 4$ and non α (n α)) which show extensive homology to the rat brain clones (Nef et. al., 1988). Moreover, the chick $\alpha 4$ and nα mRNA's are very abundant in the cerebrum, optic lobe and cerebellum in the adult and in ovo (day 9-12) and this has allowed the isolation of full length cDNA clones for these genes. Messenger RNA's encoding the chick brain $\alpha 2$ and $\alpha 3$ clones are not as abundant in avian brain tissue and cDNA's have not yet been obtained for these genes.

1.11 Insect AChR's

Acetylcholine has long been known to be the major excitatory neuortransmitter in insect CNS (Colhoun, 1963; Pitman, 1971; Gerschenfeld, 1973; Kehoe and Marder, 1976). Three types of AChR have been identified in insect brain based on their pharmacological specificities (Sattelle, 1980) and have been classified as mixed affinity AChR's, mAChR's and nAChR's.

1.11.1 The mixed AChR

The presence of AChR's with mixed muscarinic-nicotinic pharmacology was demonstrated in soluble extracts of housefly (Musca domestica) heads by the binding of the cholinergic ligand (^{3}H)muscarone (Eldefrawi and O'Brien, 1970; Eldefrawi et. al., 1970, 1971; Cattell and Donnellan, 1972; Donnellan et. al., 1975; Mansour et. al., 1977). Both nicotinic and muscarinic ligands were shown to inhibit this binding and therefore this site was concluded to be an AChR with both muscarinic and nicotinic properties. Other radiolabelled cholinergic ligands (nicotine, decamethonium, dimethyl-d-tubocurarine and atropine) were shown to bind to this same extract (Eldefrawi et. al., 1971), although αbungarotoxin was found to have no effect on the binding of (^{3}H)decamethonium (Mansour et. al., 1977; Tripathi et. al., 1979). Various attempts were made to purify this protein from the 100,000 x g supernatant (Jewess et. al.,

1975; Mansour et. al., 1977; Tripathi et. al., 1979; Harris et. al., 1981) and the native Mr of the protein was calculated to be 320,000 - 380,000, consisting of two types of subunit of Mr 83,000 and 91,000. Amino acid analysis of the protein revealed that it contained a high proportion of hydrophilic and polar residues suggesting that it may be a peripheral membrane protein (Harris et. al., 1981). Both types of subunit were shown to be glycoproteins with mannose, glucose and galactose being the major constituents (Donnellan and Harris, 1977; Harris et. al., 1980). The functional significance of this putative AChR is unclear and no further work has been carried out since (^{3}H)decamethonium ceased to be commercially available.

1.11.2 The insect mAChR

Muscarinic AChR's were shown to be present in membrane fractions of fruitfly heads (Dudai and Ben-Barack, 1977; Haim et. al., 1979), housefly heads (Jones and Sumikawa, 1981; Shakler and Eldefrawi, 1981), the supraoesophageal ganglion of Locusta migratoria (Breer, 1981) and Schistocerca gregaria (Aguilar and Lunt, 1984) and the cockroach (Periplaneta americana) ventral nerve cord (Lummis and Sattelle, 1985) using the muscarinic antagonist (^{3}H)quinuclidinyl benzilate ((^{3}H)QNB). Solubilisation of this binding site has proved to be difficult since the protein is particularly unstable

losing binding activity with a half-life of 24 hours at 4°C (Dudai, 1980). Triton X-100 was found to inactivate the binding activity while 2M NaCl was found to release only 20% of the total binding activity (Haim et. al., 1979). More recently, Venter et. al. (1984) affinity labelled the mAChR in fruitfly heads with (^{3}H)propylbenzylcholine mustard and identified a polypeptide of Mr 80,000 which is in close agreement with the results of radiation inactivation studies which gave a Mr of 77,600 (Lummis et. al., 1984). Monoclonal antibodies against rat brain mAChR's were shown to strongly crossreact with the fruitfly receptor (Venter et. al., 1984) and it is becoming clear that mAChR's are highly conserved throughout diverse groups of organisms. A functional role for an insect mAChR has been demonstrated at the neuromuscular junction on the extensor tibiae muscle of the metathoracic leg of Schistocerca gregaria (Fulton and Usherwood, 1977; Fulton 1982).

1.11.3 The insect nAChR

The presence of α bungarotoxin binding sites in insect CNS was first demonstrated by autoradiography of (^{125}I) α bungarotoxin binding to frozen sections of the fruitfly heads (Hall and Teng, 1975). Subsequently, (^{125}I) α bungarotoxin was shown to bind to the particulate fraction of fruitfly heads and not to the supernatant suggesting that this site was distinct from the mixed AChR (Schmidt-

Nielsen et. al., 1977; Dudai, 1977, 1978; Rudloff, 1980; Jiminez and Rudloff, 1980). Binding sites for α bungarotoxin have also been identified in housefly head homogenates (Eldefrawi and Eldefrawi, 1980; Harris et. al., 1979; Cattell et. al., 1980; Jones et. al., 1981). More recently, α bungarotoxin binding sites have been identified in locust ganglia (Filbin et. al., 1983; Breer, 1981a), cockroach CNS (Lummis and Sattelle, 1985) and honey bee heads (Sherby et. al., 1986). The binding characteristics for all these different tissues are very similar and are summarised in table 1. The binding of α bungarotoxin shows no co-operativity indicated by a hill coefficient of 0.96 whereas that for acetylcholine was calculated to be 0.5 indicating either negative co-operativity or heterogeneity of sites (Dudai, 1980). The pharmacology of binding to these binding sites in different tissues is consistent with classical nAChR's in that the binding is inhibited by nicotinic ligands but not by muscarinic ligands (see for review Sattelle, 1980; Breer and Sattelle, 1987). It is interesting to note that decamethonium, the ligand used to identify the mixed affinity AChR, had no effect on the binding of α bungarotoxin to fruitfly head tissue (Schmidt-Nielsen et. al., 1977). Therefore it is clear that the insect CNS does indeed contain high levels of α bungarotoxin binding sites which show a pharmacological similarity to the vertebrate nAChR.

1.11.4 Purification of the insect nAChR

Considerable effort has been made to purify the nAChR from insect tissue making use of the techniques developed for the vertebrate nAChR. This involves solubilising the component from the membrane with a detergent and purifying it on an affinity column usually made with α bungarotoxin covalently linked to a gel support such as Sepharose. The α bungarotoxin binding component has been solubilised from the membrane fraction of fruitfly heads and exhibits similar binding characteristics and pharmacological specificities to that observed in the membrane bound form (Schmidt-Nielsen et. al., 1977). However, Dudai (1978) reported difficulties in solubilising the fruitfly head α bungarotoxin binding component with Triton X-100 and even in the presence of NaCl only partial release of the binding activity was observed. A more efficient extraction from this tissue was achieved with deoxycholate at pH 9.0 (Jimenez and Rudloff, 1980). Moreover, a purification scheme giving a 1200 fold purification of the α bungarotoxin binding site from fruitfly heads was reported (Gepner, 1979; Hall, 1980), using a combination of Triton X-100 (1% W/V) and NaCl (0.1 M) followed by affinity chromatography on an α cobratoxin-Sepharose 4B affinity column. The pharmacology of binding to this purified component was similar to that of the membrane bound form and the estimated Mr of the receptor-toxin complex was 500,000 by gel filtration and 300,000 by

sucrose density sedimentation, The α bungarotoxin binding site has been purified from housefly heads using affinity chromatography on an α bungarotoxin affinity column and on denaturing gel electrophoresis consisted of subunits of Mr 42,000 and 25,000 with an intact Mr of 200,000 while the 42,000 subunit was found to label with MBTA (March et. al., 1982). α Bungarotoxin binding components have also been purified from two species of locust. The protein purified by Filbin et. al. (1983) from Schistocerca gregaria was found to consist of polypeptides of Mr 60,000, 41,000 and 25,000 on denaturing gel electrophoresis with a polypeptide of Mr 58,000 being labelled with MBTA. In contrast, the protein purified from Locusta migratoria was found to consist of one major subunit type with a Mr of 65,000, although a minor component with Mr 58,000 was often observed and attributed to proteolysis (Breer et. al., 1984). The intact protein was calculated to have a Mr of 250,000 - 300,000 by non-denaturing polyacrylamide gel electrophoresis (Breer and Sattelle, 1987) and was therefore proposed to be a homooligomer made up from four or five of the 65,000 Mr subunits representing an evolutionarily primitive form of the nAChR. It was proposed that the gene coding for this 65,000 Mr subunit was an ancestral gene from which all other nAChR's have evolved (Breer et. al., 1985). Affinity labelling of this putative nAChR has not been reported. More recently, the α bungarotoxin binding

component in cockroach CNS has been purified and was found to be identical to that of Locusta with one major subunit of 65,000 on denaturing gel electrophoresis (Sattelle and Breer, 1985). Therefore it is clear that there is no consistency in the results from different groups for the molecular composition of the insect nAChR with much work remaining to be done to clarify the status of this receptor.

1.11.5 Distribution of (^{125}I)α bungarotoxin binding sites

To satisfy the criteria for identification of a specific binding component as a receptor it is necessary to examine the localisation of the binding site and correlate it with synaptic activity. The first demonstration of non-uniform distribution of αbungarotoxin binding in the insect nervous system was shown by Hall and Teng (1975) in fruitfly with the binding being localised only to neuronal tissues. This binding was subsequently shown to be confined to synaptic areas of the neuropile and was absent from nerve tracts and cell bodies in the fruitfly CNS (Schmidt-Nielsen et. al., 1977; Dudai and Amsterdam, 1977; Rudloff, 1978) and the binding within the neuropile region was of non-uniform distribution. This localisation of αbungarotoxin binding to specific neuropile regions was also observed in the moth (Manduca sexta) with only a small level of binding being detected over non-synaptic regions of the CNS such as the non-synaptic neuropile, the

cell body regions and the antennal nerves (Hildebrand et. al., 1979). The 6th abdominal ganglion of the cockroach has also been shown to bind α bungarotoxin in the central neuropile region, although some binding was present in the periphery of the ganglion where glial cells and neuronal cell bodies are present (Sattelle et. al., 1981, 1983). The pharmacology of this binding in all these studies was consistent with a nAChR. The binding of α bungarotoxin to fruitfly brain was blocked with unlabelled toxin and d-tubocurarine whereas atropine reduced but did not block completely the binding (Schmidt-Nielsen et. al., 1977; Rudloff, 1978). Nicotine was also found to block α bungarotoxin binding in autoradiographic studies (Dudai and Amsterdam, 1977). Similarly, the binding observed in the moth preparation was blocked by acetylcholine (in the presence of neostigmine) and d-tubocurarine but was only slightly reduced by QNB (Hildebrand et. al., 1979). Therefore, there is good evidence in support of the insect α bungarotoxin binding component being a functional nAChR situated in a synaptic location. However, in contrast to this, there is evidence to suggest that α bungarotoxin fails to block cholinergic transmission in insect CNS (see section 1.11.7).

1.11.6 Antibodies as probes of the insect nAChR

Antibody studies are useful both in determining similarities between receptors from different species and

in localising receptors in tissues. Monoclonal antibodies against the Torpedo marmorata nAChR have been shown to cross react with a protein in the neural membrane of the locust (Fels et. al., 1983). Two of these antibodies were directed against a region close to or at the ligand binding region of the nAChR since they could compete against α bungarotoxin binding. Furthermore, the single subunit of Mr 65,000 purified from the locust ganglia was identified in a Western blot with one of these monoclonal antibodies (Breer et. al., 1985) thereby confirming the cross-reactivity of the locust receptor with the Torpedo receptor. A monospecific antiserum raised against the nAChR purified from Locusta was used to identify receptors in the neuropile region of head and thoracic ganglia of the locust (Breer et. al., 1985) and the 6th adominal ganglion of the cockroach (Sattelle, unpublished but see Breer and Sattelle, 1987). Monoclonal antibodies raised against the Torpedo californica nAChR, all specific for different epitopes, were shown to crossreact with fruitfly neural tissue identifying different regional subsets of receptor in axonal tracts, neuropile region, mechano-sensory bristle elements and photoreceptors (Chase et. al., 1987). One of these antibodies has been used to immunoprecipitate a protein which has a Mr of 51,000 on denaturing gel electrophoresis. An auto-anti-idiotypic acetylcholine antibody was used in an immunocytochemical localisation study in Locusta migratoria where specific

immunoreactivity was identified in the neuropile areas of the protocerebrum, the optic lobes, the deutocerebrum, and the tritocerebrum of the supraoesophageal ganglion of Locusta migratoria (Vieillemaringe et. al., 1987).

1.11.7 Evidence for functional insect nAChR's

It is necessary to back up radiolabelled binding studies with evidence that the binding site has a functional role in vivo. Most evidence for the insect α bungarotoxin binding site representing a functional nAChR has come from electrophysiological studies on the cockroach 6th abdominal ganglion (for review see Breer and Sattelle, 1987). This has been the tissue of choice due to the easily identifiable synapses of the cercal afferent and giant interneurone pathway. Iontophoretic application of ACh onto the cell body and synaptic regions of the giant interneurone 2 resulted in membrane depolarisations (Callec, 1974; Callec et. al., 1982; Harrow and Sattelle, 1983). Moreover, α bungarotoxin was found to block synaptic transmission in the cercal afferent giant interneurone pathway (Sattelle et. al., 1983). The time taken to achieve block was significantly greater when presynaptic release of acetylcholine was increased either by increased stimulation frequency or by the presence of 4-aminopyridine, and when anticholinesterases were present. The concentration of αbungarotoxin required to produce blockade of synaptic transmission was found to be

close to its calculated Kd (Sattelle, 1986). The muscarinic antagonist QNB could not block synaptic transmission in this system confirming that the cholinergic response at this synapse is nicotinic in nature. The cell body of giant interneurone 2 also contains nAChR's as demonstrated by iontophoretic application of ACh onto the cell body which resulted in a depolarising response that could be blocked by α bungarotoxin but not by QNB (Harrow and Sattelle, 1983). Voltage clamp studies have yielded valuable information on the properties of the ion channel. The AChR of the fast coxal depressor motorneurone (Df) of the cockroach has been studied using this technique (Harrow et. al., 1982) showing that this receptor gates a cation channel permeable to sodium, potassium and possibily calcium ions. Voltage clamp experiments have also revealed information on antagonist classification. For example, α bungarotoxin causes a block of receptor function in a voltage independent manner (David and Sattelle, 1984) whereas histrionicotoxin (Sattelle and David, 1983), amantadine (Artola et. al., 1982) and d-tubocurarine (David and Sattelle, 1984) were shown to be voltage dependent blockers. The insecticide nereistoxin was shown to suppress acetylcholine induced currents in a voltage dependent manner (Sattelle et. al., 1985). In contrast, an α bungarotoxin-insensitive nAChR has been identified on the dorsal unpaired median neurone cell body

of the grasshopper (Schistocerca nitens) (Goodman and Spitzer, 1979) and in cockroach metathoracic ganglia (Sattelle et. al., 1980) although the pharmacological specificity of these receptors has not yet been reported and their function is unclear.

More recently the technique of patch clamping has been applied to the study of insect nAChR's allowing detailed analysis of the channel. A single class of inward channel was identified on cell bodies of cockroach ganglia showing a single channel conductance of 40 pS and a reversal potential of 60 mV positive to resting potential (Sattelle et. al., 1986). The purified nAChR of Locusta migratoria was reconstituted into planar lipid bilayer membranes and single channel recordings of this reconstituted receptor gave a conductance of 70 - 80 pS (Hanke and Breer, 1986). Therefore, a functional role for insect neuronal nAChR's has been demonstrated in several insect species by a variety of techniques.

1.11.8 Cloning of insect nAChR's

Following the work on vertebrate nAChR's, progress is now being made on the isolation of genes encoding insect nAChR's. Functional expression of mRNA extracted from ganglia of Locusta has been achieved in the oocyte expression system (Breer and Benke, 1985). Injection of increasing amounts of total mRNA resulted in an increase

in the number of α bungarotoxin binding sites synthesised. These binding sites were labelled during synthesis with (^{35}S)methionine, solubilised and precipitated with an antiserum prepared against the purified nAChR from Locusta. On denaturing gel electrophoresis a similar subunit pattern as for the purified nAChR was observed with this precipitated material (Breer and Benke, 1986). Additionally, these receptors have been shown to be functional nAChR's by ion flux studies (Breer and Benke, 1986). Hermans-Borgmeyer et. al., (1986) have isolated two overlapping cDNA clones (ARD) from a cDNA library of fruitfly heads using cDNA probes of the α and γ subunits of the Torpedo nAChR. The mature encoded protein is predicted to consist of 497 amino acids and the sequence shows considerable homology to other nAChR subunits, and is thought to be a non α subunit since it lacks the two adjacent cysteine residues corresponding to 192 and 193 of the Torpedo receptor α subunit. A similar clone was isolated from a genomic library of the locust (Schistocerca gregaria), using cDNA probes for the chick brain nAChR (Marshall et. al., 1988a). This clone (non-alpha) also has considerable homology to other nAChR subunits as well as to the ARD fruitfly clone. More recently, another insect nAChR subunit gene has been identified by screening a fruitfly genomic library with the chick α2 probe (Bossy et. al., 1988). Restriction fragments containing exons of this gene were further used

to probe Drosophila instar cDNA libraries resulting in the isolation of three overlapping cDNA clones coding for an alpha like subunit (ALS) of the nAChR. The expressed ALS-β-galactosidase fusion protein failed to bind αbungarotoxin and was therefore proposed to be similar to the vertebrate neuronal α subunits. Additionally, this ALS clone, the locust non-alpha clone and the fruitfly ARD clone all show greater homology to the vertebrate neuronal nAChR subunits than to the vertebrate neuromuscular α subunits.

1.12 Receptor Superfamilies

The full coding sequences of the two subunit types of the $GABA_A$ receptor (Schofield et. al., 1987) and the strychnine binding subunit of the glycine receptor (Grenningloh et. al., 1987) have recently been reported and show extensive homology (about 50%) to each other and about 25% homology to each of the subunits of the Torpedo nAChR. Further, all these receptor subunits contain the same pattern of four transmembrane domains. It has been suggested, therefore, that there is a family of genes coding for ligand gated ion channels which may have evolved from an ancestral gene. This can be compared to the second type of neurotransmitter receptor, those linked to second messenger systems, which also represents a superfamily of receptors such as various adrenergic, muscarinic and opsin types (Dixon et. al., 1986; Nathans and Hogness, 1983). In the nAChR, a region of major

interest is an extracellular β-structural loop which can be formed by the disulphide bonding of two conserved cysteine residues. This region shows very high homology in all the subunits of the Torpedo receptor as well as to the vertebrate neuronal subunits and the insect cDNA clones as shown in figure 8. The invariant proline residue in this sequence is predicted to form a β hairpin turn resulting in a β structure with one face completely hydrophobic. This region is present in all ion channel receptors and it has been proposed to be part of the ligand binding site of the nAChR (Luyten, 1986).

1.13 The Aim of This Project

The true molecular structure of the insect nAChR is still largely unclear and controversial with different results emerging from the various groups of study. This project has attempted to define more clearly the pharmacological characteristics of the locust nAChR, to improve the purification of this protein, to make an immunological study of the locust nAChR and to provide information that may help in the cloning of insect nAChR genes. It seems likely that the true subunit structure of the insect nAChR will be elucidated by the techniques of molecular biology, although it will be essential to back up the cloning work with biochemical evidence. The results presented here are a step in this direction.

CHAPTER 2

2.1 Materials

Adult locusts (Schistocerca gregaria) were purchased from Cambridge Biosciences Services (Chesterton Hall Crescent, Cambridge, UK) and Larujon Ltd. (Welsh Mountain Zoo, Colwyn Bay, Wales). (-)-(N-methyl-^{3}H)nicotine (78 Ci/mmol) and (^{125}I)Na were purchased from Amersham International (Amersham, Bucks, UK). (N-methyl-^{3}H)methylcarbamylcholine iodide (^{3}H)MCC) (86.6 Ci/mmol) was purchased from Du Pont (UK) Ltd. (NEN Research Products, Wedgewood Way, Stevenage, UK). (+) and (-) anatoxin-a were a gift from Dr H Rapaport (Dept. of Chemistry, University of California, Berkley, USA) and MLA was a gift from Dr M H Benn (Dept. of Chemistry, University of Calgary, Alberta, Canada). (+)-Nicotine di(-)tartrate was provided by Dr R Barlow (Dept. of Pharmacology, University of Bristol, Bristol, UK) and dihydro β erythroidine (DHβE) was provided by Dr R G Benfield (Merck Sharp & Dohme, Hoddesdon, Herts, UK). Mab 270, Mab 290 and Mab 299 were provided by Dr J Lindstrom (Salk Institute, San Diego, U.S.A.). Amino acid derivatives for the peptide synthesis were purchased from Cambridge Research Biochemicals Ltd. (Button End, Harston, UK). All other peptide synthesis reagents and 1,1-Dimethyl-4-piperazinium (DMPP) were purchased from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, UK). HPLC grade acetonitrile was purchased from BDH Ltd. (Broom Road, Poole, Dorset, UK). HPLC grade water was purified on a

Millipore Milli-Q reagent grade water system. Nitrocellulose paper was purchased from Sartorius Ltd. (Avenue Road, Belmont, Surrey, UK). Optiphase "safe" was purchased from LKB Instruments Ltd. (Addington Road, Selsdon, South Croydon, UK). All other drugs and chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Tritium was measured in either a LKB 1217 Rackbeta or a Packard Tricarb 4000 series liquid scintilation counter and ^{125}I was measured in a LKB 1280 ultrogamma counter.

2.2 Tissue Preparation

Two week old adult locusts were used for all experiments. Insects were anaesthetised with CO_2, decapitated and the heads stored on ice. The cuticle was shaved off, from the mouth, through the eyes to the top of the head and the supraoesophageal ganglion removed to aluminium foil on ice. For the binding experiments, as much of the eye material was removed as possible but in the case of the purification experiments the eye material was included. One hundred ganglia without eyes have a wet weight of approximately 250 mg.

2.3 Tissue Fractionation

2.3.1 Preparation of a P2 Membrane Fraction From Locust Ganglia

The preparation of the P2 membrane fraction was essentially as previously described by Filbin et. al. (1983). The fractionation scheme is shown in figure 9. Ganglia were homogenised in 50 mM potassium phosphate buffer, pH 7.4 (10 ml per 100 ganglia), containing 0.25 M sucrose, 1 mM EDTA, 1mM EGTA and 0.01% (w/v) sodium azide at 4°C, using a Potter-Elvejham homogeniser with a motor driven pestle (speed, 500 rpm; 30 passes; radial clearance, 0.15 mm) The homogenate was filtered through nylon bolting cloth (159 μm mesh) and the filtrate centrifuged at 500 x g for 10 minutes at 4°C in a Sorval RC5B centrifuge with an SS34 rotor. The supernatant was retained on ice and the pellet resuspended in the same buffer and recentrifuged. The combined supernatants were centrifuged at 100,000 x g for 30 minutes at 4°C in a Beckman L5-50B ultracentrifuge with a SW50.1 rotor. This pellet was termed the P2G pellet and was resuspended in 50 mM potassium phosphate buffer, pH 7.4, at a final ratio of 6 ganglia per ml for the (^{3}H)(-)nicotine and (^{125}I) α bungarotoxin assays and 12 ganglia per ml for the (^{3}H)MCC assay. For some experiments the P2G fraction was washed in either 50 mM potassium phosphate buffer, pH 7.4, or distilled water by resuspending in 30 ml and standing at 4°C for 60 minutes. The membrane was then pelleted by centrifugation at 100,000 x g as described above.

2.3.2 Preparation of a P2 Membrane Fraction From Whole Heads

The whole heads were homogenised in a Sorval Omnimixer (3 x 20 seconds, speed setting 5) at 4°C and the homogenate filtered through nylon mesh. The filtrate was further homogenised and fractionated exactly as described in section 2.3.1 with the exception that an S35 rotor replaced the SW50.1 rotor. The final pellet was termed the P2H pellet.

2.3.3 Preparation of a Detergent Extract From Locust Ganglia

Figure 10 shows the fractionation scheme for the preparation of the detergent extract. The ganglia were homogenised in 10 mM potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, 1mM EGTA and 0.1% (w/v) sodium azide (10 ml per 100 ganglia), as previously described (section 2.3.1). The homogenate was filtered through nylon mesh and the filtrate centrifuged at 45,000 x g for 30 minutes at 4°C in a Sorval RC5B centrifuge with an SS34 rotor. The resulting pellet was resuspended in 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 3%(w/v) lubrol PX and 0.01%(w/v) sodium azide and incubated for 30 minutes at 20°C with constant mixing. The mixture was then centrifuged as before and the resulting supernatant was termed the detergent extract.

2.4 Iodination of αBungarotoxin

α Bungarotoxin was iodinated to a specific activity of around 700 Ci/mmol (depending on the batch), according to the method of Urbaniak et. al. (1973). α Bungarotoxin (2.5 nmol) in 20 µl of 50 mM potassium phosphate buffer, pH 7.5, was mixed with 20 µl of carrier-free Na ^{125}I (100 mCi/ml) in dilute NaOH solution and 10 µl of 50 mM potassium phosphate buffer, pH 7.5. The reaction was started by the addition of 10 µl of 0.5% (w/v) chloramine-T in 50 mM potassium phosphate buffer, pH 7.5 and allowed to proceed for 1 minute at 20°C with constant stirring. The reaction was terminated by the addition of 750 µl of 0.016% (w/v) sodium metabisulphite in 50 mM potassium phosphate buffer, pH 7.5 and 200 µl of 1% (w/v) potassium iodide in 50 mM potassium phosphate buffer, pH 7.5. (^{125}I) α bungarotoxin was separated from free ^{125}I by passage down a Sephadex G25 column (25 cm x 1 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.4, containing 1% (w/v) bovine serum albumin (BSA) and eluted with the same buffer. Fractions (1 ml) were collected and the radioactivity in 10 µl aliquots was determined. The peak fractions were pooled and the specific activity of the (^{125}I) α bungarotoxin was calculated assuming 100% recovery of the protein. The toxin was stored at 4°C in elution buffer and was used for up to 4 weeks.

2.5 Radioligand Binding Assays

2.5.1 Assay of (^{3}H)(-)Nicotine Binding to the P2G Fraction

Tissue samples (0.25 ml) were incubated with (^{3}H)(-) nicotine (50 nM unless otherwise stated) for 30 minutes at 20^{o}C, in the presence and absence (10 minute preincubation) of excess unlabelled (-)nicotine (10^{-3}M) to determine non-specific binding. In competition assays, tissue samples were preincubated with serial dilutions of the drug for 10 minutes at 20^{o}C prior to addition of radioligand. It should be noted that preincubation may lead to desensitisation of the receptor. The samples were chilled on ice (60 minutes), diluted with 2 ml ice-cold 50 mM potassium phosphate buffer, pH 7.4, and rapidly filtered under vacuum on Whatman GFC filters presoaked in 0.3% (v/v) polyethyleneimine (PEI) (Bruns et. al., 1983). The filters were washed twice with 2 ml of 50 mM potassium phosphate buffer, pH 7.4; filtration and washing was accomplished within 30 seconds. The filters were transferred to scintillation vials, 5 ml of Optiphase "safe" scintillant was added and the radioactivity determined.

2.5.2 Assay of (^{125}I) αBungarotoxin Binding to the P2G Fraction

The binding of(^{125}I) α bungarotoxin to the P2G fraction was measured in essentially the same way as for (^{3}H)(-) nicotine. Tissue samples (0.25 ml) were preincubated for

10 minutes at 20°C in the presence and absence of excess unlabelled α bungarotoxin (10^{-6}M) or serial dilutions of competing ligand before addition of (^{125}I) α bungarotoxin (1 nM unless otherwise stated). Incubation was continued for 60 minutes at 20°C and the samples were filtered as for the (^{3}H)(-)nicotine assay.

2.5.3 Assay of (^{3}H)MCC Binding to the P2G Fraction

The binding of (^{3}H)MCC to the P2G fraction was determined exactly as for (^{3}H)(-)nicotine binding (section 2.5.1.), with the exception that the membrane fraction contained twice the protein concentration.

2.5.4 Assay of (^{125}I) α Bungarotoxin Binding of to the P2H Fraction

Aliquots (100 μl) of the P2H fraction were incubated with (^{125}I) α bungarotoxin (20 nM) for 60 minutes at 20°C. Incubation was terminated by addition of 1 ml of ice-cold 50 mM potassium phosphate buffer, pH 7.4, and the samples were centrifuged at high speed in an MSE microcentaur. The pellets were resuspended in 1 ml of ice-cold 50 mM potassium phosphate buffer, pH 7.4, and recentrifuged. The pellets were then counted for radioactivity. Non-specific binding was determined in the presence of an excess of unlabelled α bungarotoxin (10^{-6}M).

2.5.5 Assay of (^{125}I) α Bungarotoxin Binding to the Detergent Extract and the Purified α Bungarotoxin Binding Component

Aliquots (100 μl) were incubated, in the presence and absence of an excess of unlabelled αbungarotoxin (10^{-6}M), with (^{125}I) α bungarotoxin (10 nM) at 20^{o}C for 60 minutes. The samples were then filtered on Whatman GFB filters that had been presoaked in 0.3% (v/v) PEI. The filters were washed with 5 ml of 10 mM potassium phosphate buffer, pH 7.4, containing 0.1% (w/v) Lubrol PX and counted for radioactivity.

2.6 Affinity Purification of the α Bungarotoxin Binding Component

2.6.1 Preparation of the α Bungarotoxin-Sepharose 4B Affinity Gel

The affinity gel was prepared according to the method of March et. al. (1974). Sepharose 4B beads (25 ml packed beads) were washed with 500 ml of 0.1 M NaCl followed by 500 ml of distilled water. The beads were resuspended in 50-100 ml of distilled water at 4^{o}C and 100 ml of 2 M di-sodium carbonate at 4^{o}C was added. Cyanogen bromide (CNBr) in acetonitrile was added to a final concentration of 50 mg CNBr per ml of beads and stirred for 2 minutes at 4^{o}C. The beads were filtered rapidly and washed with 500 ml of distilled water at 4^{o}C over a period of 2-3 minutes.

α Bungarotoxin (2 mg) in 25 ml of 0.2M sodium hydrogen carbonate, pH 9.4, was added to the activated Sepharose-4B and mixed at 4°C for 21 hours. The beads were then washed with 400 ml of distilled water (pH 9.0 with potassium hydroxide). 1M glycine (200 ml) was added to the beads and mixed for 2 hours at 20°C. The Sepharose 4B was washed with 150 ml of 0.1 M sodium acetate, pH 4.0, followed by 150 ml of 0.2 M sodium hydrogen carbonate, pH 9.4. This was repeated another twice and the affinity beads were finally resuspended and stored in 10 mM potassium phosphate buffer, pH 7.4, containing 0.002% (w/v) thimerosal as preservative and stored at 4°C.

2.6.2 Purification of the αBungarotoxin Binding Component

The detergent extract (section 2.3.3.) was mixed with 1-2 ml (packed bed) of the α bungarotoxin-sepharose 4B for 4-6 hours. The beads were then packed into a column 0.5 x 10 cm. Non-bound material was washed out with two washes of 2 ml of 10 mM potassium phosphate buffer, pH 7.4, containing 0.5% (w/v) Lubrol PX and 1 M NaCl followed by 20 ml of 10 mM potassium phosphate buffer, pH 7.4, containing 0.5% (w/v) Lubrol PX. Bound protein was eluted from the affinity column onto a DE52 ion exchange column by recycling 4 mM benzoquinonium chloride overnight (Lindstrom et. al., 1981). The DE52 ion exchange column was washed with 50 ml of 10 mM potassium phosphate buffer,

pH 7.4, containing 0.5% (w/v) Lubrol PX. Protein was recovered from the DE 52 column by elution with 10 mM potassium phosphate buffer, pH 7.4, containing 0.5% (w/v) Lubrol PX and 1 M NaCl. Fractions were collected (0.25 ml) and assayed for the binding of (^{125}I) α bungarotoxin (see section 2.5.5).

2.7 Analysis of the Purified α Bungarotoxin Binding Component

2.7.1 Precipitation of the α Bungarotoxin Binding Component

The purified α bungarotoxin binding component was precipitated using a chloroform/methanol method (Wessel and Flugge 1984). Briefly, 4 parts methanol and 1 part chloroform were added to 1 part sample, followed by 3 parts water. Phase separation was achieved by centrifugation in a MSE microcentaur, and the upper phase was removed. Three parts methanol were added and the protein pelleted by centrifugation. The supernatant was removed and the pellet dried under a stream of nitrogen.

2.7.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was carried out under denaturing conditions according to the method of Laemli (1970). Pellets of the α bungarotoxin binding component and the P2G fraction were resuspended in sample buffer (0.0625 M

tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.02% (w/v) bromophenol blue, 5% (v/v) glycerol), boiled for 5 minutes and allowed to cool before being loaded onto the gel. Electrophoresis was performed in a Bio Rad Protean II system with a 10% running gel and 5% stacking gel under constant current of 16 mA for the first 45 minutes and then 24 mA until the dye front was approximately 1 cm from the bottom.

2.7.3 Gel Protein Staining

2.7.3.1 Silver stain

Gels were stained using the method of Morrisey (1981). All solutions were made in double distilled water. The gel was fixed in 10% (w/v) trichloroacetic acid overnight at 20°C, followed by extensive washing at 20°C with 10% (v/v) ethanol and 5% (v/v) acetic acid (3 x 30 minutes). The gel was then incubated in oxidiser solution (0.0034 M potassium dichromate, 0.0032 N nitric acid) for 60 minutes at 20°C followed by 2 x 5 minute washes at 20°C with double distilled water. The gel was then incubated with 0.15% (w/v) silver nitrate at 20°C for 60 minutes in the dark, and washed once with double distilled water. Protein bands were developed with 3% (w/v) di-sodium carbonate containing 0.0185% (v/v) formaldehyde at 42°C until the required image developed. Development was terminated by transfer of the gel to 5% (v/v) acetic acid for 5 minutes. Gels were stored in double distilled water.

2.7.3.2 Coomassie blue stain

Gels were fixed and stained simultaneously with 0.25% (w/v) coomassie brilliant blue R250 in 45% (v/v) methanol and 9% (v/v) acetic acid overnight at 20°C. Destaining of the gel was achieved with 30% (v/v) ethanol and 10% (v/v) acetic acid. Gels were stored in 10% (v/v) acetic acid.

2.8 Affinity Labelling of the P2G Fraction

The P2G fraction was labelled with the affinity ligand 4-(N-maleimido)-benzoyltrimethylammonium iodide (MBTA) by the method of Barrantes et. al. (1975). Tritium labelled MBTA was synthesised by Dr R Harrison by the method of Karlin et. al. (1971) to a specific activity of 126 μCi/mol. The P2G fraction was reduced by resuspending the pellet in 0.5 ml of 1 mM dithiothreitol in 10 mM tris-HCl buffer, pH 7.3, containing 0.1 M NaCl and 1 mM EDTA, and incubated for 10 minutes at 20°C. The samples were centrifuged in a MSE microcentaur at high speed for 5 minutes. The pellets were resuspended in 0.5 ml of 10 mM potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA, and recentrifuged. The pellets were resuspended in the same buffer and (-) nicotine (10^{-3}M) was added to one of the samples, to determine non-specific binding, and incubated for 60 minutes at 20°C. (^{3}H)MBTA was dissolved in 0.1 mM HCl and 1 nmol was added to each sample and incubated for 3 minutes at 20°C. Excess β mercaptoethanol (10 μl) was added to stop the reaction and the samples immediately centrifuged. The pellets were

washed once with 10 mM potassium phosphate buffer, pH 7.4, and finally resuspended in electrophoresis sample buffer. Electrophoresis was performed as detailed in section 2.7.2. After electrophoresis the gel was stained with coomassie brilliant blue as detailed in section 2.7.3.2. Each track of the gel was then sliced (6 slices/cm) and the slices dried and solubilised in 500 µl of 1% (v/v) ammonium hydroxide in 30% (v/v) hydrogen peroxide. Optiphase "safe" (5 ml) was added and the samples counted.

2.9 Peptide Synthesis

2.9.1. Chemical synthesis

The peptide was synthesised on a CRB Pepsynthesiser II controlled by an Olivetti M24 PC. Synthesis was based on the Fmoc-polyamide chemistry. This method uses derivatised amino acids with a pentafluorophenyl (Pfp) group on the carboxyl group and a 9-fluoromethoxycarbonyl (Fmoc) protecting group on the amino group of the amino acid (Arshady et. al., 1981; Atherton et. al., 1981). The resin used was the pepsyn KA type (figure 11). The solvent used was N,N-dimethyl formide (DMF) which was distilled under reduced pressure prior to use and stored in a dark bottle for up to a week. This procedure removed any amines which would interfere with the synthesis. An outline of the synthesis procedure is shown in figure 12. The initial coupling step was carried out with recirculation for 4 hours in the presence of the catalyst

4-dimethylaminopyridine. The Fmoc protecting group was not removed and the coupling procedure was repeated overnight to achieve maximum coverage of the resin. The Fmoc group was then removed with the base piperidine (20% (v/v) in DMF). Subsequent amino acids were added in the presence of 1-hydroxybenzotriazole and recirculation was for approximately 25 minutes. After recirculation, a small sample of the resin was taken and tested for free amino groups.

2.9.1.1 Kaiser colour test for the detection of free amino groups

A small sample of the resin was washed twice with DMF, twice with dichloromethane (DCM), twice with ether and dried under a stream of nitrogen. To the beads, in a small tube, one drop each of 5% (v/v) ninhydrin in ethanol, 400% (w/v) phenol in ethanol and 20 μM potassium cyanide in pyridine. The sample was heated for 5 minutes at 100^{o}C. A blue colouration in the beads indicated incomplete coupling whereas a yellow colouration indicated complete coupling.

2.9.1.2 Colour test for the detection of free proline amino groups

A slight modification was introduced to the Kaiser test for the detection of free proline amino groups. The beads were washed as in the previous colour test and two drops

of a saturated solution of istatin in benzyl alcohol containing 5% (w/v) Boc-Phe-OH was added. Two drops of each of the Kaiser colour test solutions were added and incubated at 100°C for 5 minutes. The beads were then washed with acetone. Complete coupling was indicated by white beads whereas a red/blue colouration indicated incomplete coupling.

2.9.1.3 Completion of the synthesis

After the final amino acid was added, the protecting group was removed. The resin was washed with 25 ml tertiary amyl alcohol (TAA), 25 ml glacial acetic acid, 25 ml TAA, 25 ml DCM and 50 ml ether. The resin was then dried under a stream of nitrogen and the dried resin was stored at -20°C.

2.9.1.4 Cleavage of the peptide from the resin

The resin was incubated in a solution of 5% (v/v) ethylmethylsulphide (EMS) in trifluoroacteic acid (TFA). EMS was included as a scavenger for the protection of methionine in the peptide. This solution was collected by passage through a sinter and subjected to rotary evaporation. Residual EMS was removed with a water/ether separation system where the peptide separated into the water phase and the EMS into the ether phase. The water phase was washed a further two times with ether and the final water phase freeze dried.

2.9.2 Analysis of the Peptide

2.9.2.1 High performance liquid chromatography (HPLC)

HPLC was performed on a Milton Roy LDC system equiped with two constaMetric III metering pumps, a spectroMonitor III variable wavelength detector, a solvent mixer unit, a Rheodyne injection valve and a plotter all controlled by an LDC Chromatography Control Module. A reverse phase Hypersil WP-300 butyl 10μ column (250 mm x 5 mm id) was used with a guard column of the same material (50 mm x 5 mm id). The solvent system consisted of water and acetonitrile (both containing 0.1% (v/v) TFA) with a gradient from 5% (v/v) to 60% (v/v) acetonitrile at a rate of 2% change per minute. A flow rate of 1 ml per minute was used.

2.9.2.2 Amino acid analysis

Amino acid analysis was performed on a Hilger Analytical Chromaspek II. Peaks observed on the HPLC trace were collected and dried under vacuum in acid washed glass tubes. The samples were resuspended in 6N HCl ("Aristar") and sealed under vacuum. Hydrolysis was carried out at 105^{o}C for 24 hours. The samples were dried down under vacuum and then resuspended in 25 mM HCl prior to loading into the analyser.

2.10 Raising Antisera to the Synthetic Peptide

2.10.1 Immunisation

Californian white rabbits were used for all immunisations. The peptide, dissolved in phosphate buffered saline (PBS), was emulsified in Freunds complete adjuvant for initial immunisations and Freunds incomplete adjuvant for subsequent boosts. Immunisations were performed intramuscularly in two sites with 100 μg peptide per rabbit. Boosts were made at two week intervals. Serum samples were taken seven days after each boost. Antiserum 109 was collected by total bleed after two boosts.

2.10.2 Assaying the Antisera

Blood samples were allowed to clot at 37^{o}C for 60 minutes and transferred to 4^{o}C overnight. Samples were then centrifuged at 3000 rpm for 15 minutes in an MSE bench centrifuge. The supernatant was removed and stored at -40^{o}C. Antisera were assayed for positive responses to the peptide using a dot immunobinding assay (Hawkes et. al., 1982). Aliquots (1 μ l) were spotted onto nitrocellulose filters and allowed to dry. The filters were transferred to 4^{o}C for 10 minutes to stabilise the binding and washed in PBS for 5 minutes. The filters were blocked with a solution (1 ml per filter) of 1% (w/v) casein in PBS (blocking buffer) for 30 minutes at 20^{o}C with constant agitation. Antiserum dilutions were made up in blocking buffer containing 5% (v/v) normal goat serum

(NGS). The primary antibody incubation (1 ml per filter) was carried out overnight at 20°C with constant agitation. The filters were washed (3 x 10 minutes) with blocking solution (2 ml per filter) and incubated for 2 hours at 20°C with a 1/1000 dilution of goat anti rabbit IgG - horse radish peroxidase conjugate (1 ml per filter) with constant agitation. The filters were then washed with blocking buffer (2 ml per filter) and a final wash of PBS (2 ml per filter) prior to development. A solution (1 ml per filter) of 50 mM sodium acetate, pH 5.0, containing 0.05% (w/v) 3-amino-9-ethyl carbazole and 0.03% (v/v) hydrogen peroxide was added to the filters and incubated at 20°C until the colour developed, usually about 15 minutes.

2.11 Western Blotting

Electrophoresis was performed as described in section 2.7.2. To identify tracks on the nitrocellulose sheet, methyl green was added, during electrophoresis, to the gel tracks approximately half way through the run and then again 10 minutes from the end. After electrophoresis, protein was transferred to nitrocellulose paper at 30 volts for 20 hours at 4°C with 25mM potassium phosphate buffer, pH 7.4, containing 10% (v/v) methanol as the blotting buffer. The nitrocellulose sheet was washed (4 x 15 minutes) with PBS (100 ml) containing 0.01% (v/v) Tween 20 for 30 minutes at 20°C. Individual tracks were cut out

and immunostained essentially as previously described in section 2.10.2 except that PBS containing 0.2% (w/v) casein and 0.05% (v/v) Tween 20 was used as blocking buffer. Protein standards transferred to the nitrocellulose sheet were stained with indian ink. The track containing the standards was washed (2 x 30 minutes) at 20°C with PBS (10 ml) containing 0.1% (v/v) Tween 20. The filter was then incubated overnight at 20°C with PBS (10 ml) containing 0.1% (v/v) Tween 20 and 0.1% (v/v) indian ink. This solution was poured off and the individual protein standards were immediately visible. This method has the advantage that no destaining is necessary. The filters were washed in distilled water and dried.

2.12 Precipitation of the αBungarotoxin Binding Component with Antisera and Monoclonal Antibodies

The detergent extract prepared as in section 2.3.3 was used in immunoprecipitation assays. Aliquots (300 µl) were incubated with 5-10 µl of antiserum for 2 hours at 20°C. IgG was precipitated by incubation at 4°C overnight with a goat anti rabbit IgG antiserum (100 µl per 5 µl of rabbit serum), that was raised in this department. Samples were centrifuged and the supernatant diluted with an equal volume of buffer. The diluted samples were then assayed for (^{125}I) α bungarotoxin binding activity as described in section 2.5.5. Experiments with monoclonal

antibodies were performed essentially in the same way with 100 µl of culture supernatant or 5-10 µl of concentrated supernatant. Normal serum (5 µl) was added as a carrier for precipitation by the anti-Ig antiserum.

2.13 Assay for the Expression of (^{125}I) α Bungarotoxin Binding Sites in Oocytes

Oocytes were injected with total poly A+ mRNA isolated from locust ganglia and locust whole heads (50 ng). As a positive control mRNA from Torpedo (50 ng) was used. The oocytes were allowed to incubate for 48 hours at 23°C prior to assay for specific binding of (^{125}I) α bungarotoxin. Oocytes were resuspended in 50 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF and 1% (v/v) triton X-100, homogenised and mixed for 60 minutes at 4°C. Non-solubilised material was pelleted by centrifugation in an MSE microcentaur at high speed for 5 minutes. The supernatant was assayed for (^{125}I) α bungarotoxin binding activity as described for the detergent extract (see section 2.5.5).

2.14 Protein Determinations

Protein was determined using the Lowry assay (1951) with the modifications suggested by Markmell et. al. (1978). Aliquots (200 µl) containing 10-100 µg protein, were mixed with 600 µl of a solution containing 2% (w/v) di-

sodium carbonate, 0.4% (w/v) sodium hydroxide, 0.16% (w/v) sodium potassium tartrate, 1% (w/v) SDS and 0.04% (w/v) copper sulphate and incubated for 15 minutes at 20°C.

Folin and Ciocalteu's phenol reagent was diluted 1:1 and 60 µl added to the each sample with vigorous mixing. The samples were incubated at 20°C for 45 minutes and the absorbance at 660 nm measured. Bovine serum albumin was used as the protein standard.

CHAPTER 3

3.1 Comparison of (^{125}I) ∝Bungarotoxin and (^{3}H)(-) nicotine Binding to the P2G Fraction

Binding assays were performed as detailed in the methods section (section 2.5).

3.1.1 The Effect of Varying the Protein Concentration on the Binding of (^{125}I) ∝Bungarotoxin

The binding of (^{125}I) ∝ bungarotoxin at a fixed concentration of 1 nM was measured over the protein concentration range 15-110 μg. This binding was found to be linear thereby suggesting that the binding is directly proportional to protein concentration. A representative experiment is shown in figure 13.

3.1.2 The Effect of Varying the Protein Concentration on the Binding of (^{3}H)(-)Nicotine

At the fixed concentration of 40 nM (^{3}H)(-)nicotine (chosen because this would be a saturating concentration in the case of rat brain), the binding was measured over the protein concentration range 15-110 μg. Again the binding was found to be proportional to protein concentration. A representative experiment is shown in figure 13. All subsequent binding experiments on the P2G fraction with (^{125}I) ∝bungarotoxin and (^{3}H)(-)nicotine were carried out at a protein concentration of 268 ± 48 μg per ml (n = 14).

3.1.3 Dependence of Binding of (^{125}I) α Bungarotoxin on Concentration

Samples were incubated with (^{125}I) α bungarotoxin over the concentration range 0.1-10 nM. A typical binding profile for specific binding is shown in figure 14. Saturation is seen to occur over this range and the Scatchard analysis (Scatchard, 1949) of these data, shown in figure 15, is consistent with a single population of sites. A dissociation constant (Kd) of 0.8 nM and a maximum number of binding sites (Bmax) of 1.2 pmoles per mg protein were calculated. The correlation coefficient was 0.98 for this Scatchard plot. Specific binding typically represented 71% of the total binding at 10 nM.

3.1.4 Dependence of Binding of (^{3}H)(-)Nicotine on Concentration

Samples were incubated with (^{3}H)(-)nicotine over the concentration range 10-100 nM. The binding profile for specific binding is shown in figure 16. Specific binding did not reach saturation even at a concentration of 100 nM and higher concentrations of ligand were impractical due to the cost. Scatchard analysis of these data again revealed a single population of sites (figure 17) although this must be interpreted with some caution since saturation of sites was not achieved. However, from these data a Kd of 130 nM and a Bmax of 4 pmoles per mg protein were calculated. The correlation coefficient for the

Scatchard plot was 0.827. Specific binding represented 65% of the total binding at 100 nM.

3.1.5 The Effect of Washing the P2G Fraction on the Kinetics of (^{3}H)(-)nicotine Binding

The P2G fraction was washed with either 50 mM potassium phosphate buffer, pH 7.4, or distilled water as described in section 2.3.1. Neither treatment was found to have the effect of lowering either the Kd or Bmax for (^{3}H)(-) nicotine binding. Table 2 shows a comparison of the kinetic parameters calculated from the Scatchard analysis for the unwashed, water washed and buffer washed membrane preparations. The water washed P2G fraction was found to have very similar values to the unwashed P2G fraction. However, the buffer washed P2G fraction had a higher Kd and Bmax compared to the unwashed P2G fraction.

3.1.6 Determination of Ki Values for Various Nicotinic Ligands Inhibiting (^{125}I) α Bungarotoxin and (^{3}H)(-) Nicotine Binding

All the inhibition studies were performed on the unwashed P2G fraction. Table 3 shows a summary of the calculated Ki values for the various ligands studied.

3.1.6.1 Stereospecificity of nicotine inhibition

(-) and (+) nicotine were shown to inhibit (^{125}I)

αbungarotoxin and (^{3}H)(-)nicotine over the concentration range 10^{-3}-10^{-9}M. The displacement curves for the inhibition of (^{125}I) α bungarotoxin are shown in figure 18, and the calculated Ki's for (-)nicotine and (+)nicotine were 5.1 x 10^{-7}M and 1.5 x 10^{-5}M respectively. This represents a 29 fold stereospecificity in favour of the (-) enantiomer.

The displacement curve for the inhibition of (^{3}H)(-) nicotine binding is shown in figure 19. The calculated Ki's were 7.7 x x 10^{-7} M and 4.7 x 10^{-6} M for (-) and (+) nicotine respectively. This represents a stereospecificity of 6 fold in favour of the (-) enantiomer.

3.1.6.2 Inhibition of (^{125}I) α bungarotoxin binding by other nicotinic ligands

Two novel neurotoxins, MLA and (+)anatoxin-a, were shown to be potent inhibitors of (^{125}I) α bungarotoxin. The inhibition curve for MLA is shown in figure 20 and for (+)anatoxin-a in figure 21. The calculated Ki was 1.8 x 10^{-8}M for both compounds. (-)Anatoxin-a had a much lower potency for inhibiting (^{125}I) α bungarotoxin (figure 21) with a Ki of 1.0 x 10^{-4}M. DHβE was also found to be particularly potent at the α bungarotoxin site (figure 22). With a Ki value of 1.3 x 10^{-8}M it was the most potent inhibitor at this site. The vertebrate ganglionic nicotinic agonist DMPP was found to have a Ki of 3.8 x

10^{-6}M and the inhibition curve is shown in figure 23. High concentrations of atropine were found to inhibit (^{125}I) α bungarotoxin (figure 24) and the calculated Ki was 6.3 x 10^{-4}M. TEA was also found to have a slight effect with a Ki of 1.8 x 10^{-4}M (figure 25). αBungarotoxin was not tested in this study but previous studies in this laboratory determined a Ki of 5.3 x 10^{-9}M (e.g. Filbin et. al., 1983).

3.1.6.3 Inhibition of (^{3}H)(-)nicotine binding by other nicotinic ligands

With the exception of nicotine itself, all the nicotinic ligands tested were virtually ineffective at inhibiting the binding of (^{3}H)(-)nicotine to the P2G fraction (Table 3). DHβE (figure 22), DMPP (figure 23) and MLA (figure 20) had Ki values of 1.8 x 10^{-4}M, 2.0 x 10^{-4}M and 2.5 x 10^{-4}M respectively. The Ki values for (+) and (-) anatoxin-a (figure 21) were $>10^{-5}$M and $>10^{-3}$M respectively. Even αbungarotoxin was found to be only a weak inhibitor of (^{3}H)(-)nicotine binding with a Ki of $>10^{-6}$M (figure 26). The Ki for TEA was calculated to be $>10^{-3}$M (figure 25) ruling out the possibility that this nicotine site may just be binding any quaternary ammonium ion. The classical muscarinic antagonist atropine was found to have a Ki of 8.2 x 10^{-5}M (figure 24) and therefore was as potent as any of the nicotinic ligands tried. Decamethonium, the high affinity ligand used to identify

the mixed AChR in insects (see Introduction section 1.11.1), was found to have a Ki of 1.4 x 10^{-4}M (figure 26) in this preparation and therefore (^{3}H)(-)nicotine is obviously not binding to the mixed AChR in these experiments.

3.2 The Vertebrate Neuronal Nicotinic Radioligand (^{3}H)MCC Does Not Bind to the P2G Fraction

Binding assays were performed as detailed in the methods section. The membrane protein concentration used in these assays was double that used for the assay of (^{3}H)(-) nicotine and (^{125}I) α bungarotoxin binding.

3.2.1 Effect of Varying the Concentration of (^{3}H)MCC on Binding

Figure 27 shows the effect of increasing the concentration of (^{3}H)MCC over the range 5-100 nM. (-)Nicotine (10^{-3}M) was used to determine non-specific binding since (^{3}H)MCC binds specifically to the high affinity nicotine site in vertebrate brain. It is clear that the total and non-specific binding were almost exactly the same over this range. No specific binding could be calculated from the lower half of this concentration range. However, at concentrations of 75 nM and 100 nM a small specific binding could be calculated although this binding was very small when compared to the binding of either (^{3}H)(-) nicotine or (^{125}I) α bungarotoxin. This same result was obtained in two separate experiments.

3.2.2 Effect of Various Cholinergic Ligands on the Binding of (^{3}H)MCC

The effect of some classical nicotinic and muscarinic ligands on the binding of (^{3}H)MCC is shown in table 4. The inhibition is calculated as a percentage of the binding of (^{3}H)MCC in the absence of any ligand. It is evident that all of the ligands tested had no significant effect on the binding of (^{3}H)MCC. (-)Nicotine, atropine and TEA showed the highest inhibition although even this was very small. α Bungarotoxin was also found to have no effect on the binding of this radioligand. Therefore it appears that (^{3}H)MCC does not bind to either the αbungarotoxin binding site or the high affinity nicotine site over the concentration range and under the conditions tested.

3.3 Comparison of (^{125}I) α Bungarotoxin Binding to the P2H Fraction from Adult and from the Five Instar Stages

The binding of (^{125}I) α bungarotoxin was found to be linear over the protein concentration ranges tested in the P2H fraction from the adult and from the five instar stages. Figure 28 shows the linearity of (^{125}I) αbungarotoxin binding with protein concentration for the adult P2H fraction. Similar graphs were obtained for each of the five instar stages (not shown). From these data a rough estimate of the amount of binding per head compared to the amount of binding per mg of protein could be

calculated and this is shown in figure 29. As would be expected the amount of binding per head was found to increase with the stage of development, the highest binding per head being found in the adult P2H. However, the binding per mg protein was found not to vary significantly in the different stages of development. It is clear then from these data that the best stage of insect development to use for purification of the α bungarotoxin binding component is the adult stage.

3.4 Detergent Solubilisation and Affinity Purification of the α Bungarotoxin Binding Component

3.4.1 Distribution of (^{125}I) α Bungarotoxin Binding Activity in the Various Fractions During the Solubilisation Procedure

An outline of the solubilisation protocol is detailed in the methods section 2.3.3. Aliquots of each fraction were taken during the solubilisation procedure and assayed for (^{125}I) α bungarotoxin binding activity. Table 5 shows the distribution of binding activity in the different fractions during this procedure. After the initial centrifugation step, the majority of the binding activity is localised to the P1 fraction with only a small amount of activity left in the S1 fraction. This P1 pellet was solubilised in 5% (w/v) Lubrol PX and after recentrifugation the majority of the binding activity was present in the S2 fraction (the detergent extract).

3.4.2 Effect of Varying the Detergent Concentration on the Solubilisation of (^{125}I) α Bungarotoxin Binding Activity

The concentration of the non-ionic detergent Lubrol PX was varied over the range 0.1-10% (w/v) (table 6). The (^{125}I) α bungarotoxin binding activity present in the detergent extract was found to increase with an increase in detergent concentration up to a maximum at 3% (w/v) Lubrol PX. This activity was found to be very similar over the range 1-5% (w/v) Lubrol PX but started to decrease with increasing concentrations from 5-10% (w/v) Lubrol PX. A concentration of 3% (w/v) Lubrol PX was routinely used for subsequent purifications of the α bungarotoxin binding component.

3.4.3 The Binding of (^{3}H)(-)Nicotine to the Detergent Extract

The binding of (^{3}H)(-)nicotine to the detergent extract was measured at three concentrations (table 7). Very little binding was detected even at a concentration of 100 nM. This binding is not significant when compared to the binding of (^{125}I) α bungarotoxin to the same extract. However, the nicotine binding site may be very labile and difficult to assay when solubilised from the membrane and this may account for so little binding.

3.4.4 The Purified αBungarotoxin Binding Component

The αbungarotoxin binding component was affinity purified from the detergent extract as detailed in section 2.6.2. Figure 30 shows a typical elution profile from the DE52 ion exchange column in a purification experiment using 200 ganglia as starting material. The majority of the (^{125}I) αbungarotoxin binding activity was always present in one fraction. Direct elution from the affinity column with d-tubocurarine (10^{-3}M) followed by dialysis to remove the ligand was also tried. However, a greater yield of purified protein was achieved by the recirculation method onto a DE52 column. This may be explained by loss of protein during dialysis. The recirculation method was used for all subsequent experiments. Purifications were routinely carried out on either 100 or 200 ganglia. Attempts were made with various protein assay methods (Pierce assay (Smith et. al., 1985), Lowry assay (Lowry et. al., 1951), modified Lowry assay (Markmell et. al., 1978) and Bradford assay (Bradford, 1976)) to attempt to determine the quantity of protein eluted from the DE52 column. However, no method with the required sensitivity was found and problems of interfering chemicals in the preparations made the determination of protein content impossible. An estimate of the protein content could, however, be made assuming that each 49,000 dalton polypeptide (see section 3.4.5) binds one molecule of αbungarotoxin and from the number of pmoles of (^{125}I)

α bungarotoxin binding activity in the sample, it is possible to estimate the weight of protein present. Table 8 shows the number of pmoles of (^{125}I) α bungarotoxin binding activity purified from 100 ganglia in 16 different purifications along with the protein weight equivalents. The highest protein content was 1.6 μg per 100 ganglia and it is therefore not surprising that the protein assays tried were not sensitive enough to quantify the protein content.

3.4.5 Analysis of the Purified α Bungarotoxin Binding Component by SDS-PAGE

The α bungarotoxin binding component was prepared for SDS-PAGE as described in section 2.7.1. A large proportion of the detergent and salt present in the sample was removed by this procedure. Due to the low quantity of α bungarotoxin binding component that could be purified (see section 3.4.4), the normal coomassie blue staining procedure was not sensitive enough and a silver staining method was used (section 2.7.3.1). A typical gel pattern is shown in figure 31. The predominant band present has a Mr of 49,000. Occasionally other minor bands were observed, but were so faint as to be virtually impossible to record on film. However, figure 31 shows one such gel where there are faint bands with calculated Mr's of 58,000, 61,000 and 66,000. Purifications where there were other bands present as well as the main band at Mr 49,000,

are shown in figure 32 (lanes 2, 3 and 4). It is not clear whether these bands represent true polypeptides of the αbungarotoxin binding component or whether they are just minor contaminents. The only consistent band in all the purifications made, was at Mr 49,000. Figure 32 shows the consistency of the predominant 49,000 band in seven separate purifications. However, in two of these preparations an additional strong band (possibly a doublet) is seen at Mr 66,000 (lanes 3 and 4). This band at Mr 66,000 was observed a third time as shown in figure 33, lane 1. In this preparation bands were also present at 49,000, 46,000 and 44,000. An αbungarotoxin binding protein has been purified from another locust, Locusta migratoria, by Breer (1984). This protein is thought to be composed of four or five identical subunits of Mr 65,000. Therefore, the αbungarotoxin binding component was purified from Locusta migratoria ganglia, using the same procedure as for Schistocerca gregaria in this study. The subunit pattern comparison on SDS gels is shown in figure 33. The calculated Mr for the Locusta migratoria α bungarotoxin binding component was 51,000 compared to the usual 49,000 band seen for Schistocerca gregaria.

The effect of temperature, pH and the presence of protease inhibitors on the subunit pattern of the Schistocerca gregaria αbungarotoxin binding component on denaturing gels was investigated. By keeping the whole procedure for

purification at 4°C there was no difference in the subunit composition of the purified protein. Similarly there was no difference whether the protein was purified at pH 6.5, 7.4 or 8.0. The protease inhibitors pepstatin A, bestatin, leupeptin, lima bean tryspin inhibitor, soya bean tryspin inhibitor (all at a final concentration of 10 μg per ml) and PMSF (0.5 mM) were added to the buffers used in the purification procedure. Again no effect on the subunit pattern was seen by the inclusion of these inhibitors. Proteolysis certainly presents a problem in the purification of this protein as demonstrated in figure 34. This shows the denaturing gel of a sample of the purified α bungarotoxin binding protein which had been stored at 4°C for seven days compared with a freshly prepared sample. It is clear that the major band present has moved to a Mr of 43,000. The presence of proteolytic activity was also detected when the eluted fraction from the DE52 column was dialysed prior to precipitation. Figure 35 shows an example of this where most of the protein is found at the dye front indicating that the protein present has been severely proteolysed. No way was found of overcoming this proteolytic problem.

3.4.6 Attempts to Sequence the 49,000 Dalton Band

Attempts to obtain protein sequence were performed by the AFRC unit at Babraham. For the initial attempt at sequencing, the αbungarotoxin binding component in the

fraction containing highest binding activity was chloroform/methanol precipitated and resuspended in 0.1% (w/v) SDS. However, the sequencing results indicated that there was not enough protein present in the sample and that there was possibly more than one N-terminal present. In the second attempt, the α bungarotoxin binding component was purified from one thousand ganglia and sent to the AFRC unit. The result of this attempt was that the protein had a blocked N-terminal. No further attempts were made to sequence the protein.

3.5 (^{3}H)MBTA Labelling of the P2G Fraction

Figure 36 shows the gel slice profiles for total, non-specific and specific labelling of a denaturing gel of the P2G fraction labelled with the nicotinic affinity ligand (^{3}H)MBTA as detailed in the methods section. There was labelling all the way along the gel and this can be attributed to a greater amount of protein being loaded onto the gel corresponding to the total labelling sample. Using (-)nicotine to determine non-specific labelling, a predominant band was specifically labelled with a Mr of 49,000. Two other major specific peaks were observed corresponding to Mr of 205,000 and 30,000. The 205,000 band may represent the intact protomer of the receptor and the band at 30,000 may correspond to a possible proteolysis band sometimes observed on silver stained denaturing gels of the purified bungarotoxin binding component. A similar result was obtained in two experiments.

3.6 Synthesis of a Synthetic Peptide Corresponding to the Dicysteine Loop Region

The protein sequence of the peptide to be synthesised was taken from the locust non-alpha clone isolated from a genomic library of Schistocerca gregaria (Marshall et. al., 1988). This region of the polypeptide chain is very highly conserved (see Introduction and figure 8). The peptide was synthesised, as detailed in section 2.9, with cysteine residues containing an acetamidomethyl group to protect the side chain. Use of this residue has the advantage that it is difficult to remove and is therefore not removed when the peptide is cleaved from the resin. The resulting peptide is therefore linear rather than cyclised. This linear form of the peptide was synthesised since problems in achieving correct cyclisation were envisaged. An initial trial cleavage was performed (as detailed in section 2.9.1.4) on a small sample of the resin prior to treatment of the bulk.

3.6.1 HPLC analysis of the trial cleavage

The peptide was cleaved from the resin with 5% (v/v) EMS in TFA. The trial cleavage was carried out on 50 mg of resin. The peptide obtained was analysed by HPLC and the trace obtained is shown in figure 37. Two major peaks were identified at retention times of 8.06 (peak 1) and 20.87 (peak 2) minutes. A smaller third peak at 22.96 minutes (peak 3) was also detected. Samples of each of

these peaks were taken and analysed for amino acid composition.

3.6.2 Amino acid analysis of peaks 1, 2 and 3

Table 9 shows the amino acid analysis of peak 2. Peaks 1 and 3 were found not to be peptidic whereas peak 2 gave the correct amino acid composition for a peptide of the required sequence. In the amino acid analysis, the cysteine content was found to have a low value. This was expected since the cysteine residues were modified with the acetimidomethyl group. Therefore, the position of the modified cysteine in the amino acid analysis trace could be different from the unmodified cysteine position. The presence of carboxymethyl cysteine was detected and therefore could have been due to the modified cysteine residues.

3.6.3 Second cleavage

A second cleavage was performed on 200 mg of resin. The HPLC trace of a sample of this cleavage is shown in figure 38. The products of this cleavage experiment were found to be different from the initial cleavage. There was a peak at retention time 7.94 minutes and this was assumed to be the same as peak 1 from the previous trial cleavage. Two other peaks were evident at retention times 18.99 and 19.95 minutes (called peaks 4 and 5 respectively). The presence of peaks 2 and 3 was not detected in this

cleavage. Samples of peaks 4 and 5 were collected and analysed for amino acid content.

3.6.4 Amino acid analysis of peaks 4 and 5

The amino acid analysis of peaks 4 and 5 is shown in table 9. Both samples were found to be correct for a peptide with the required sequence with the exception that the value for tyrosine was very low in each sample. Therefore, both of these peptides may have been partially deprotected species of the peptide and it seems likely that it was the tyrosine residue that still contained a protecting group resulting in the low appearance of this residue in the amino acid analysis. The values for cysteine were again low again consistent with the modification by acetimidomethyl groups.

3.6.5 Further treatment of the products of cleavage 2

The peptide was retreated by repeating the cleavage process on the freeze dried sample from cleavage 2. HPLC analysis of the retreated sample is shown in figure 39. It is clear that both peaks 4 and 5 disappeared and a new peak appeared at retention time 20.76 minutes (peak 6). This HPLC trace is similar to that from the initial cleavage (figure 37). Amino acid analysis of peak 6 was correct for the required peptide (table 9) and essentially the same as for peak 2. Approximately 20 mg of peptide was recovered from the 200 mg of resin.

3.7 Antisera to the Synthetic Peptide

Four rabbits were immunised as described in section 2.10.1. All four antisera were found to be positive for reaction with the peptide and peptide conjugated to either BSA or hemoglobin, using the dot blot immunobinding assay. Figure 40 shows the assay for the antisera 109 and 110. Antisera 111 and 112 had equally strong responses.

3.8 Antibody Studies on the α Bungarotoxin Binding Protein

3.8.1 An Antiserum to the α Bungarotoxin Binding Protein Cross Reacts With the Synthetic Peptide

An antiserum (Anti-L) which was raised against the locust α bungarotoxin binding component by Colette O'Sullivan in this laboratory by repeated immunisation with small quantities of the purified protein was shown to cross react with the synthetic peptide. A typical dot blot immunobinding assay result of this is shown in figure 41 along with that for antiserum 109. Anti-L was found to be much weaker with the response being diluted out at a dilution of 1/500.

3.8.2 Do Antibodies Recognise the α Bungarotoxin Binding Component?

Various antibodies were tested for their ability to precipitate the α bungarotoxin binding component from the detergent extract. The results are shown in table 10. Anti-L was found to have a moderately high titre at around

21 nM. This means that 5 μl of the serum would precipitate 0.1 pmoles of (^{125}I) α bungarotoxin binding activity. An antiserum raised against purified Torpedo nAChR (Anti-T), raised by Colette O'Sullivan in this laboratory, had a titre of 8 nM (5 μl precipitated 0.04 pmoles of (^{125}I) α bungarotoxin binding activity) and therefore shows a small amount of crossreactivity of the locust α bungarotoxin binding protein with the Torpedo nAChR. Four monoclonal antibodies raised against the purified Torpedo nAChR in this laboratory by Susan Walsh (Mab B11, Mab C07, Mab C11 and Mab E08) were found not to precipitate (^{125}I) α bungarotoxin binding activity from the detergent extract. Additionally, Mab 270 raised against the chick brain nAChR (Lindstrom et. al. 1987a) and Mab's 290 and 299 raised against the rat brain nAChR (Whiting and Lindstrom, 1988) were unable to precipitate (^{125}I) α bungarotoxin binding activity from the detergent extract. Antiserum 109, (As 109, raised against the synthetic peptide) was also found not to be able to precipitate the (^{125}I) α bungarotoxin binding component in this assay. In these precipitation experiments, the negative controls consisted of normal rabbit serum for the antisera and normal culture medium and an anti-neurofilament monoclonal antibody for the monoclonal antibodies. As 109 was also tested for its ability to inhibit (^{125}I) α bungarotoxin binding to the detergent extract (table 11) and the P2G fraction (table 12).

However, no significant inhibition of binding was observed in either case.

3.8.3 Western Blots

The four anti peptide antisera (109, 110, 111 and 112) were tested for activity in Western blots. The preimmune serum for each antiserum was used as the control experiment. Each antiserum interacted with several bands which were determined non-specific due to their presence in the preimmune serum stained blots. Only one of the antisera, As 109, was found to detect two specific bands compared to the preimmune serum (figure 42). These specific bands had Mr's of 49,000 and 60,000. A non-specific band was also present which had a Mr of 58,000. This result was obtained in two separate experiments. As a control, As 109 was tested in a Western blot using a locust flight muscle membrane fraction as antigen. No specific bands were detected in this experiment.

The purified αbungarotoxin binding component from 400 locust ganglia was combined and split between two lanes of a denaturing gel which was subsequently blotted onto nitrocellulose as detailed in the methods section and immunostained with As 109. The only detectable band was found with a Mr of 58,000 (figure 43). In a parallel experiment a silver-stained gel of the combined purified protein showed the major band at a Mr of 49,000.

Therefore the difference in Mr may be explained by high concentrations of either salt or detergent in the protein sample prepared for Western blotting interfering with the mobility of the protein during electrophoresis. In fact during the running of the gel diffusion of the dye front was observed. For a silver stained gel purified material from only 25 ganglia is required and therefore the concentration of salt and detergent was 8 times as high in the sample for the Western blot. Therefore, it is reasonable to assume that the As 109 does in fact recognise the Mr 49,000 band characteristic of the α bungarotoxin binding component.

3.9 Expression of Locust Ganglia α Bungarotoxin Binding Sites in Oocytes

Oocytes were injected with total polyA$^+$ mRNA as detailed in the methods section. Table 13 shows the results of (^{125}I) α bungarotoxin binding experiments where oocytes were injected with mRNA extracted from locust heads and locust ganglia, and a positive control experiment where oocytes were injected with mRNA from Torpedo. Control oocytes, which were not injected, were also included. For the oocytes injected with locust ganglia mRNA, the binding was variable with the highest binding observed at 0.243 pmoles per oocyte. This was very similar to the value obtained for the oocytes injected with Torpedo mRNA. No binding to uninjected oocytes was observed in any

experiment. Therefore, it is clear that α bungarotoxin binding sites have been expressed in these oocytes directed by mRNA extracted from locust ganglia, although the levels expressed were quite low and this may be due to the low abundance of receptor specific mRNA in these mRNA samples.

FIGURES

Kingdom	Animalia
Subkingdom	Histozoa
Series	Coelomata
Phylum	Arthropoda
Class	Insecta
Subclass	Pterygota
Order	Orthoptera
Family	Acridoidea
Subfamily	Cyrtacanthacridinae
Genus	Schistocerca
Species	gregaria

Figure 1 : Classification of the desert locust.

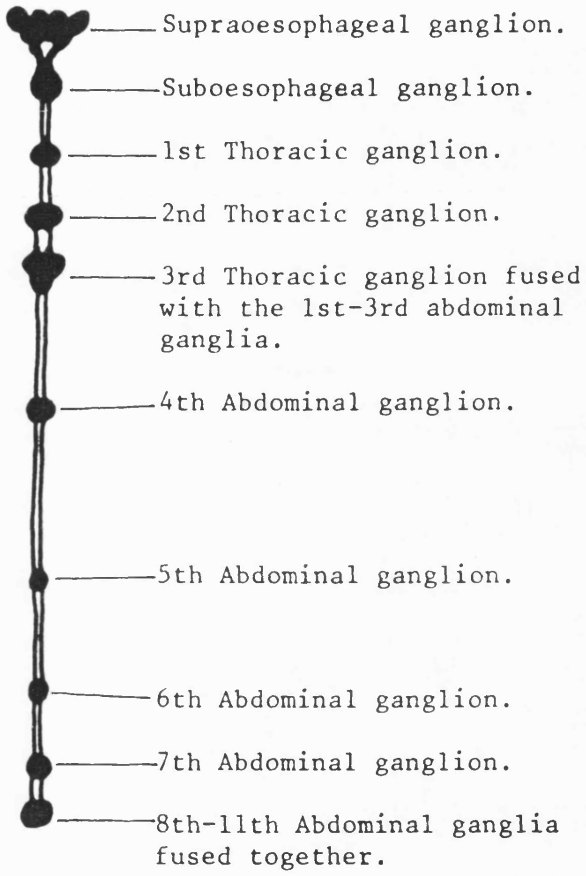


Figure 2 : The locust nervous system: arrangement of the ganglia.

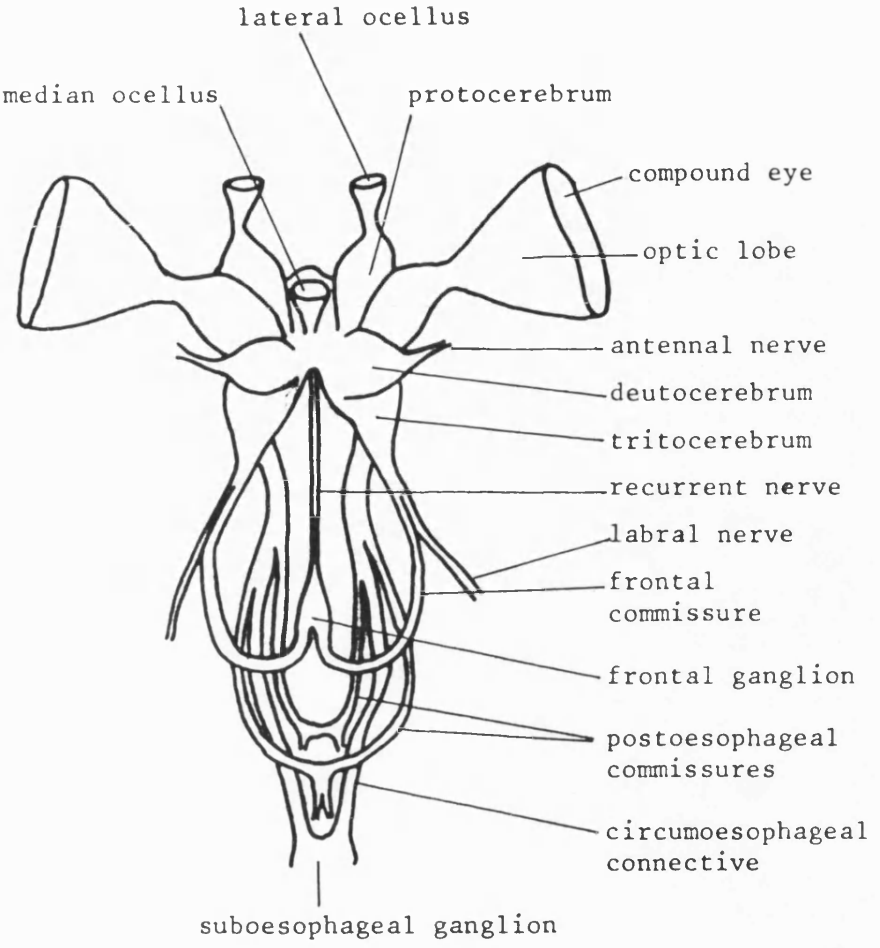


Figure 3 : Anterior view of the brain of the locust.

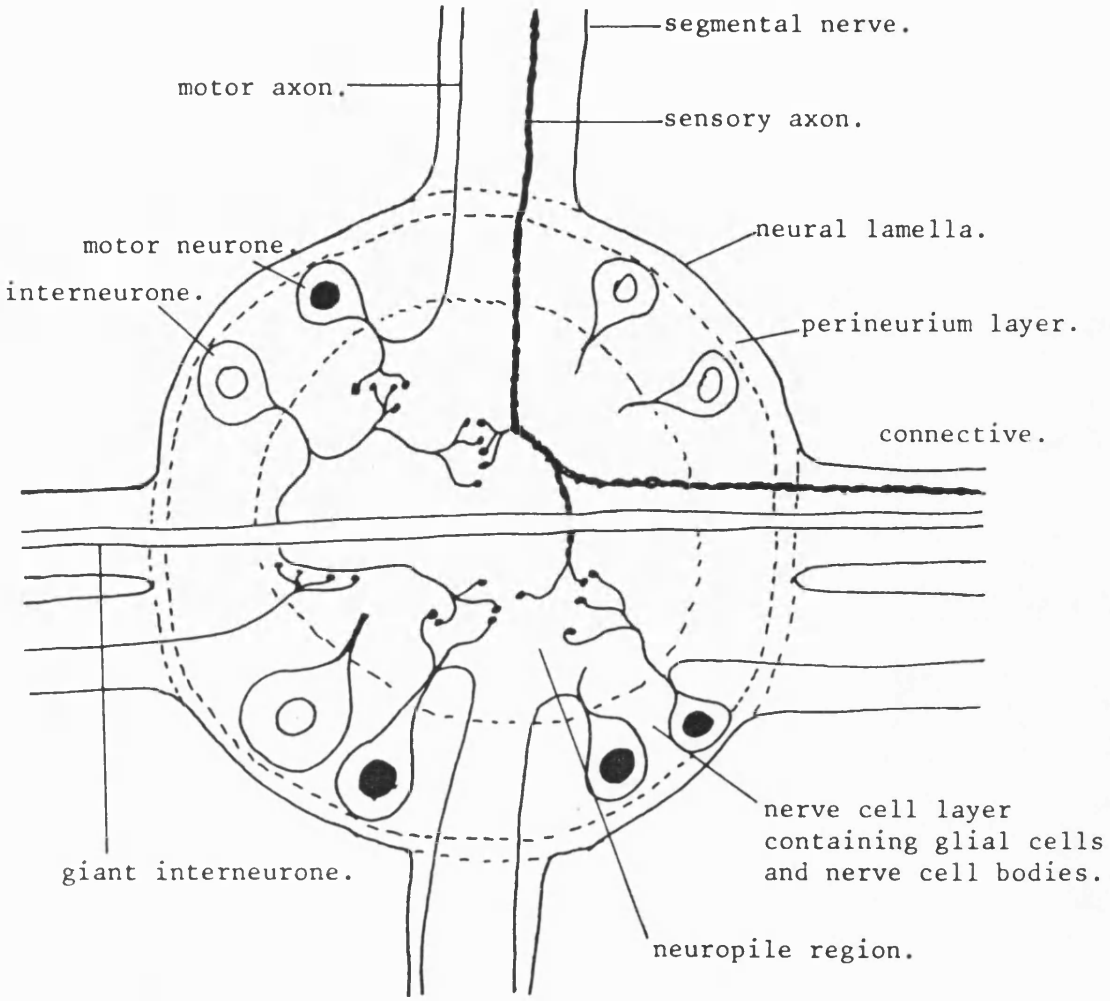


Figure 4 : Structure of a typical insect ganglion.

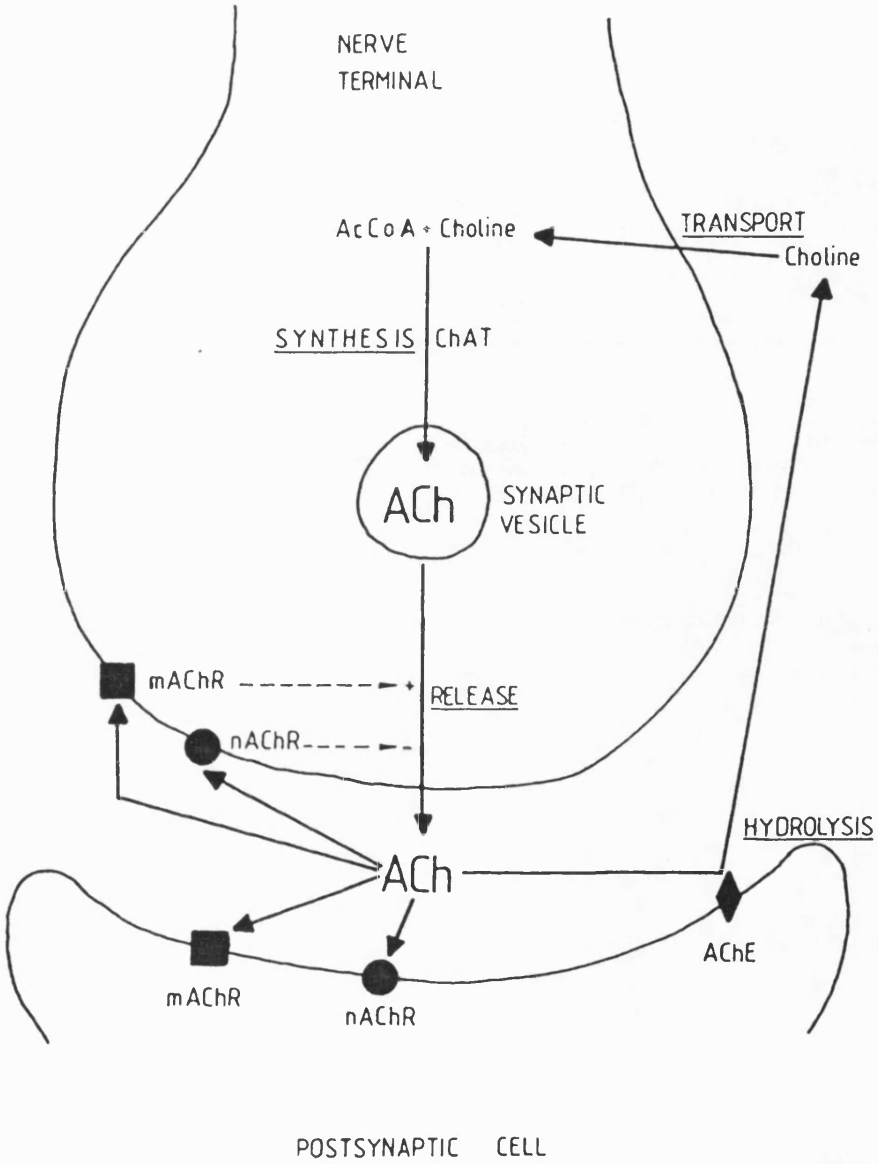


Figure 5 : Model of the cholinergic synapse.

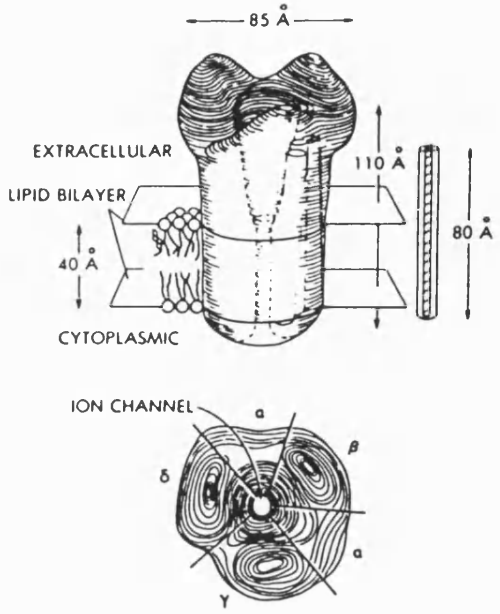
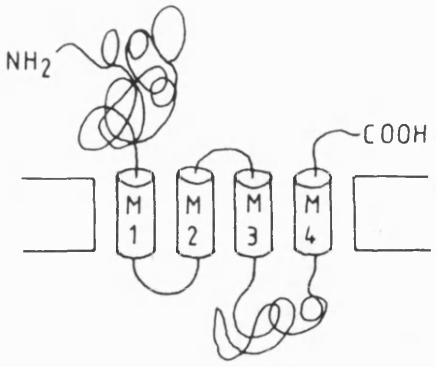


Figure 6 : A model of the three-dimentional shape of the nAChR from Torpedo californica. The positions of the subunits around the central well are tentative assignments.

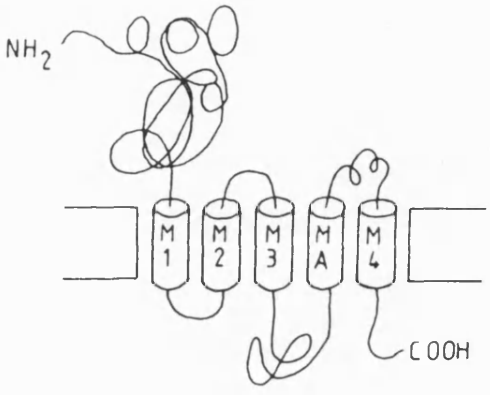
Reproduced from McCarthy et. al., 1986

Figure 7 : Three models of the arrangement of secondary structure of the Torpedo αsubunit in the membrane. Model 1 is from Noda et. al., 1982, Devillers-Thiery et. al., 1983 and Claudio et. al., 1983. Model 2 is from Guy, 1984 and Finer-Moore and Stroud, 1984. Model 3 is from Ratnam et. al., 1986b.

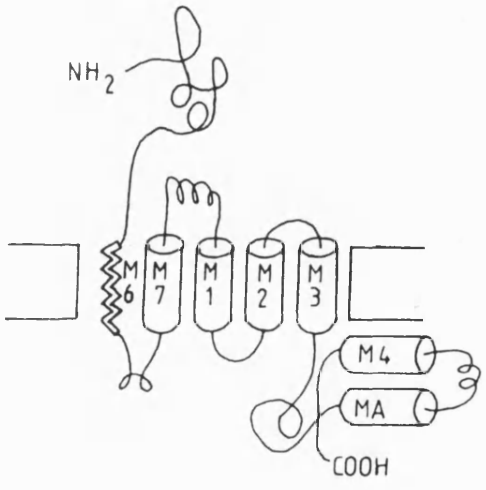
MODEL 1



MODEL 2



MODEL 3



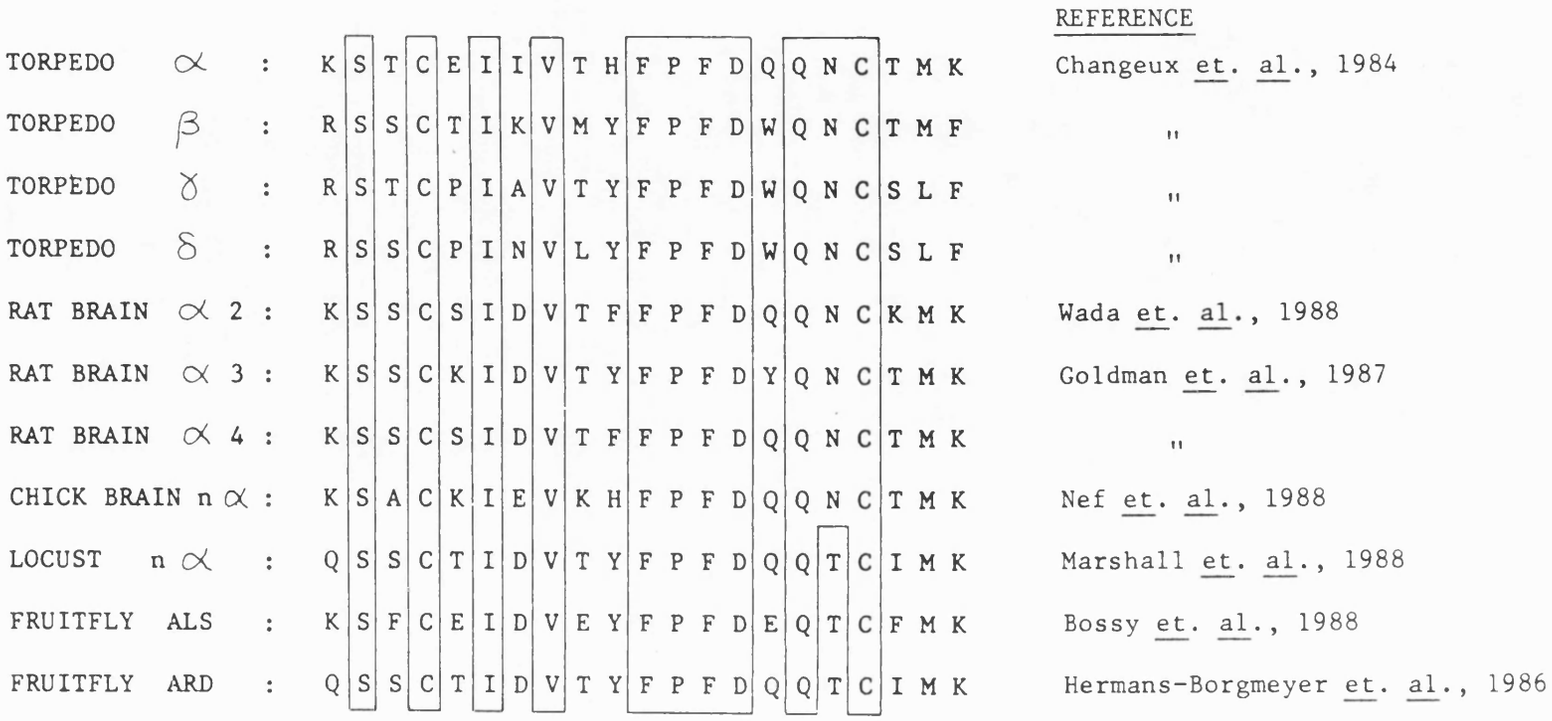


Figure 8 : Alignment of the cysteine loop amino acid sequence of the subunits of Torpedo, vertebrate brain and insect brain.

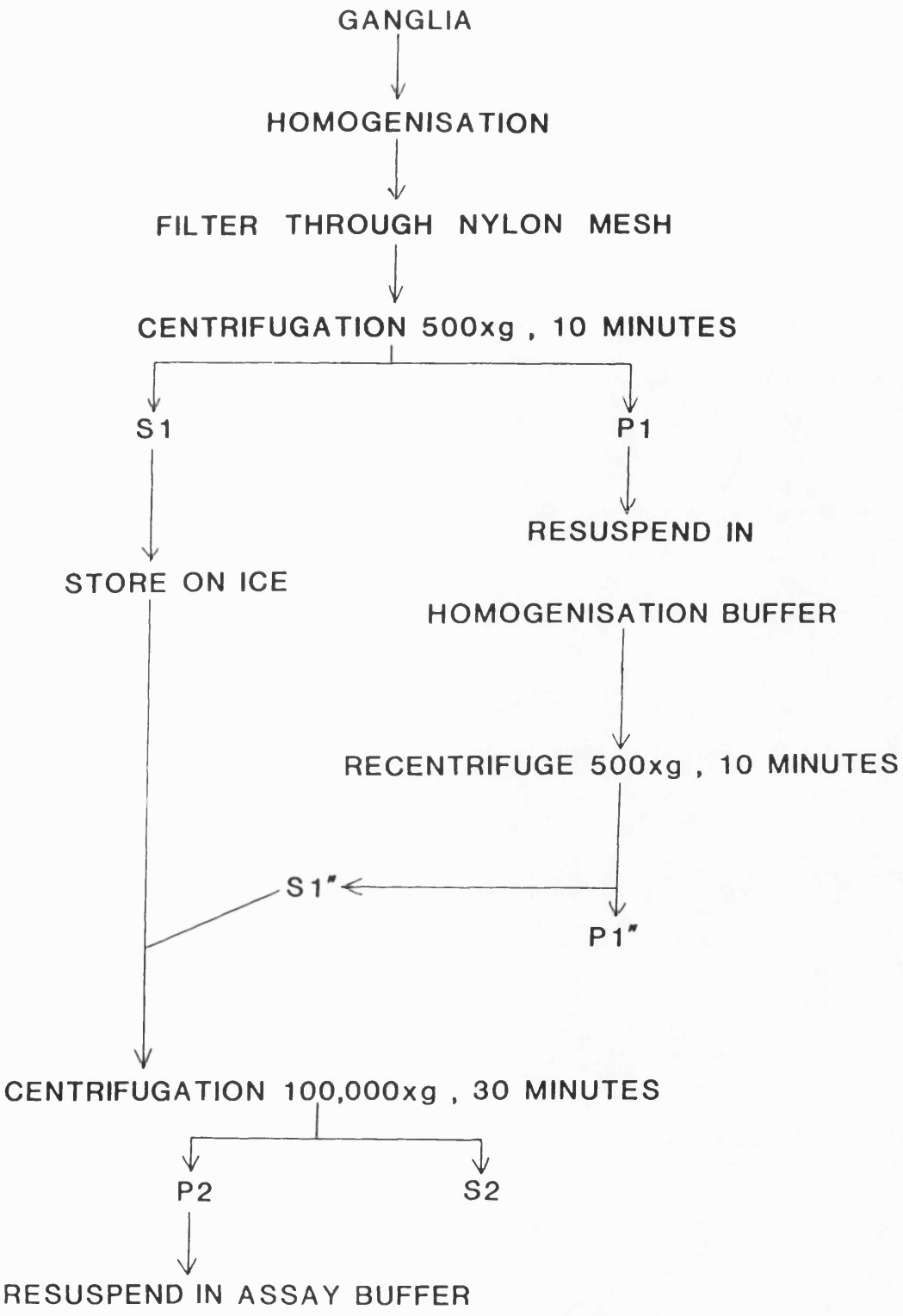


Figure 9 : Outline of the procedure for preparing the P2G fraction.

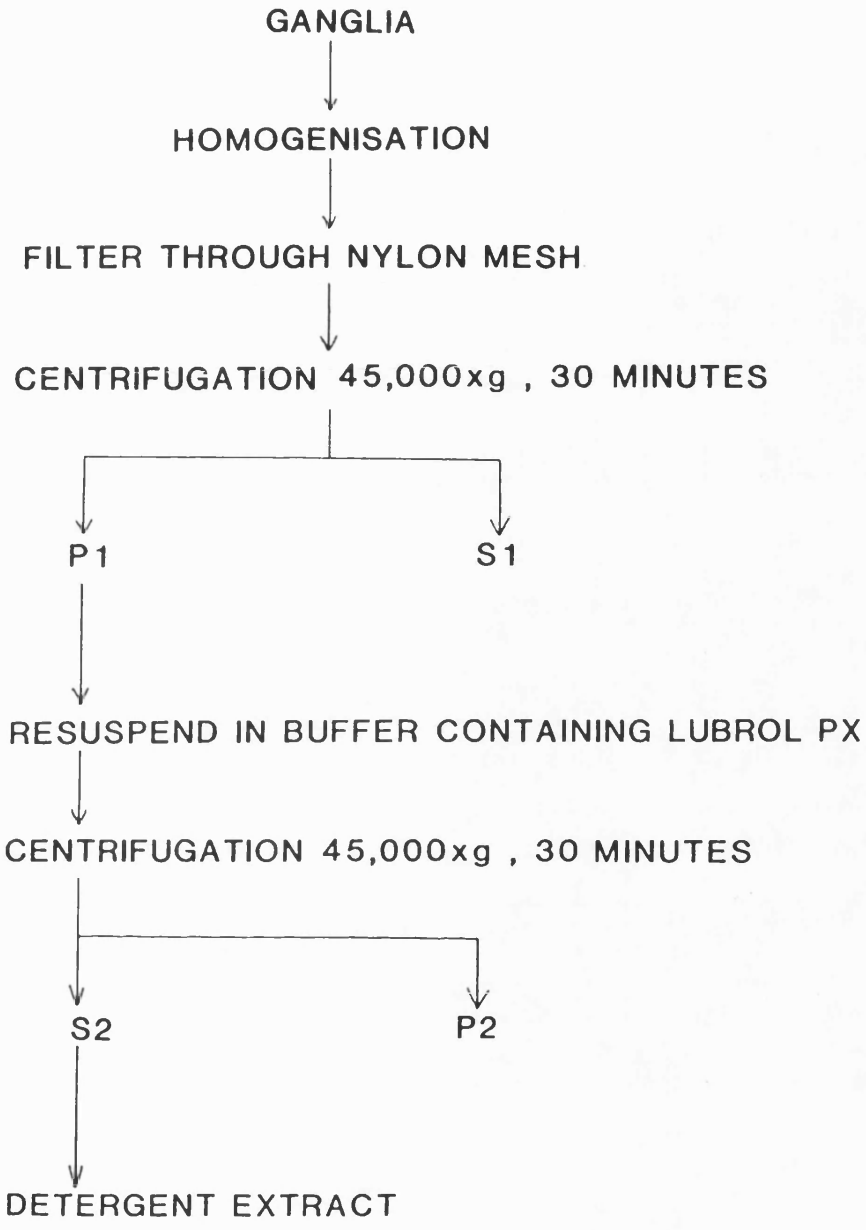


Figure 10 : Outline of the procedure for the preparation of the detergent extract.

Nle : Nor leucine

Figure 11 : Structure of the Pepsyn KA resin used for the synthesis of the peptide.

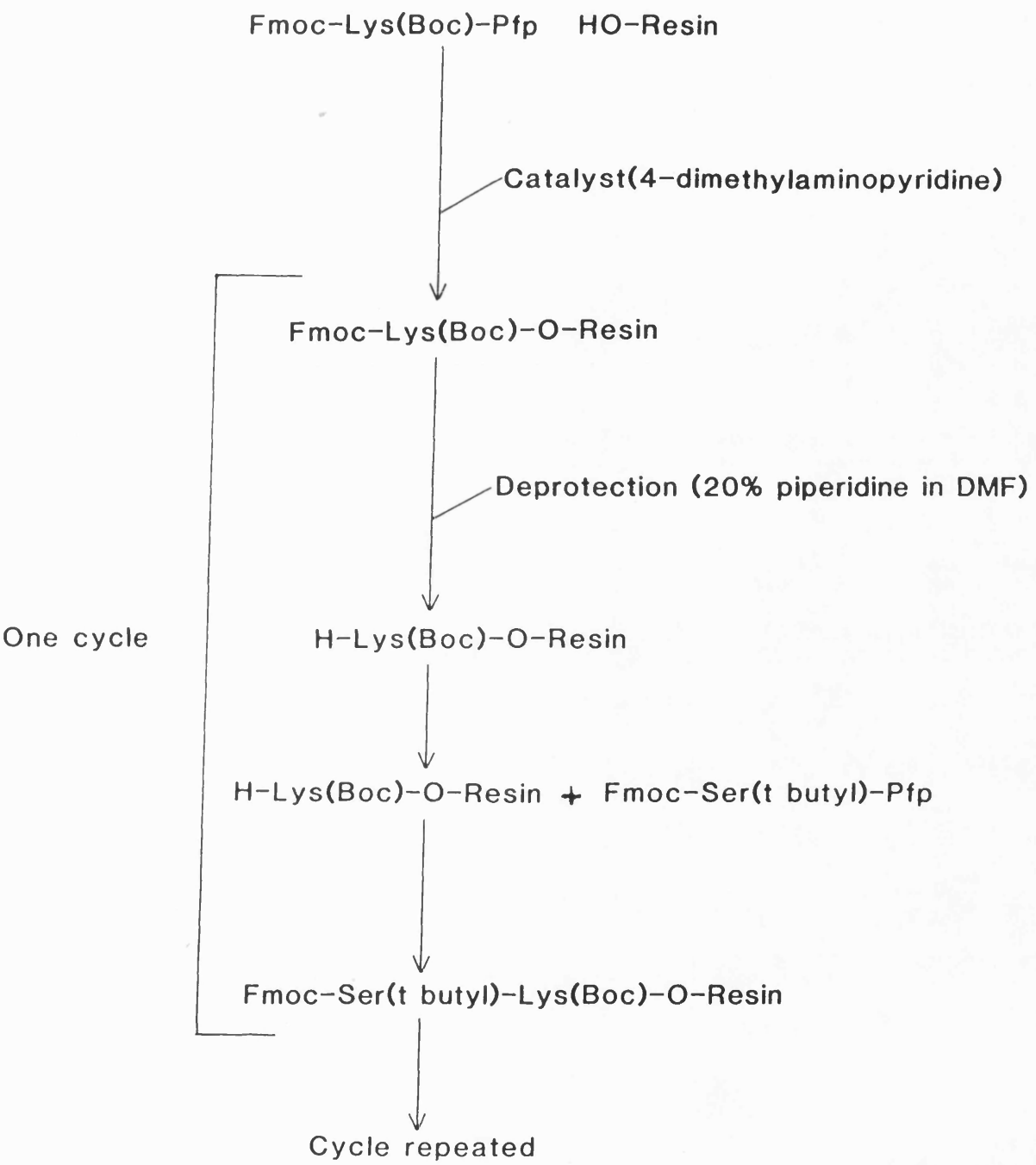


Figure 12 : Outline of the procedure for the synthesis of the peptide.

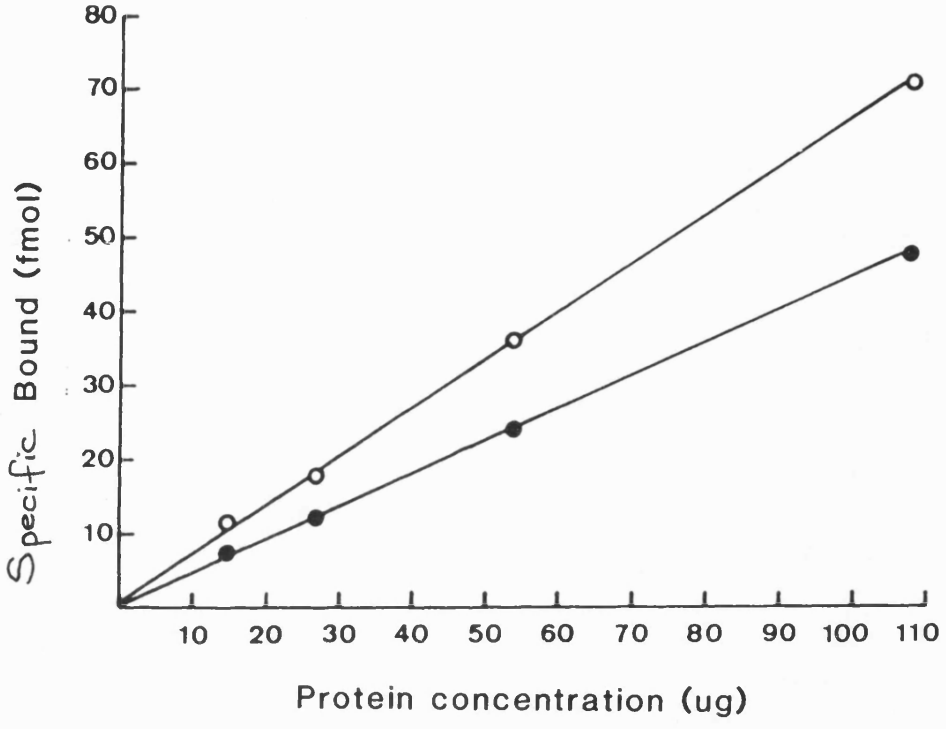


Figure 13 : Effect of varying the protein concentration on the binding of (^{125}I) α bungarotoxin (●-●) and (^{3}H)(-)nicotine (o-o) to the P2G fraction. (Results are typical of three experiments carried out in triplicate). At each protein concentration, SD < 9%.

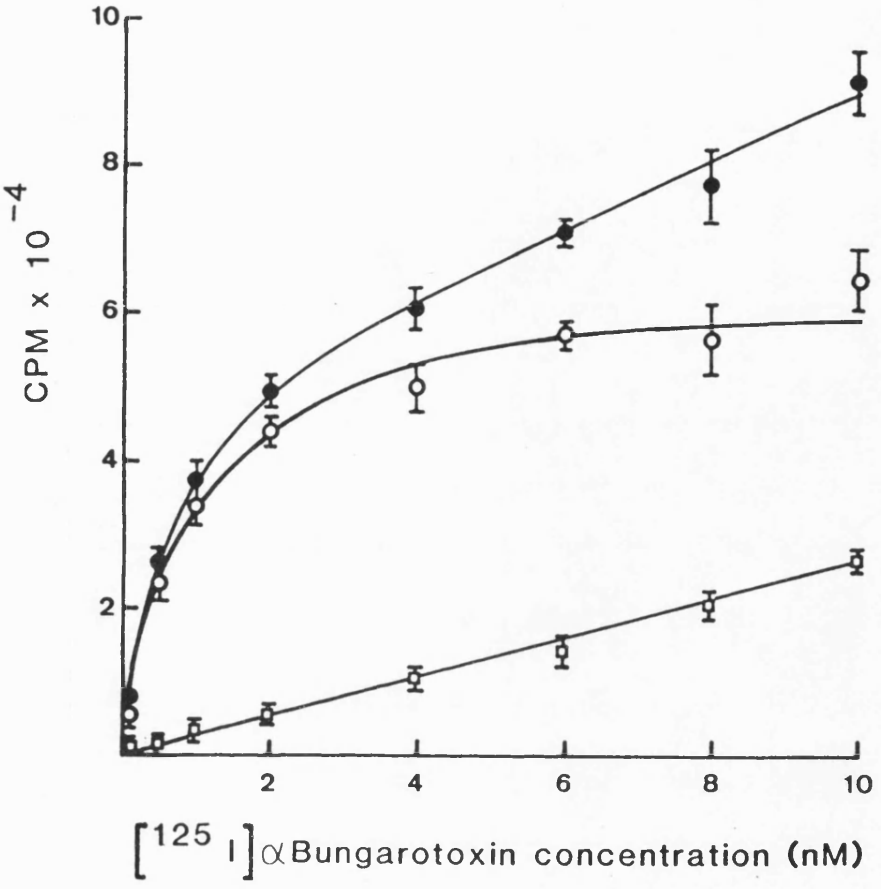


Figure 14 : The dependence of binding of (^{125}I) α bungarotoxin to the P2G fraction on concentration (0.1 - 10 nM)
(This result is from one experiment carried out in triplicate, typical of two). At each concentration, SD < 6%.

●—● Total binding
○—○ Specific binding
□—□ Non-specific binding

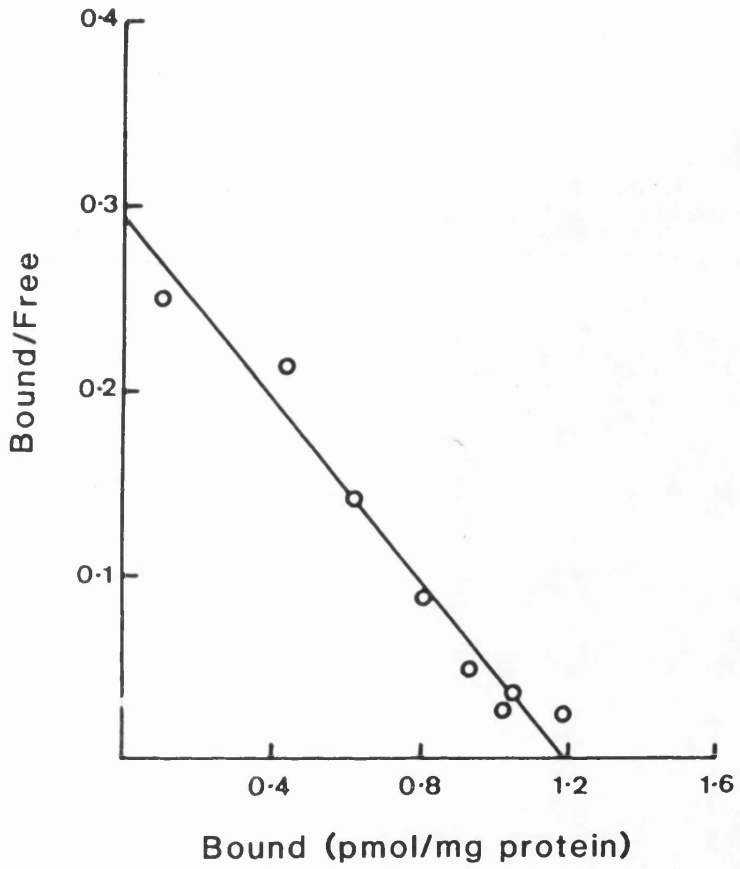


Figure 15 : Scatchard analysis of the binding data shown in figure 14. From the data a Kd of 0.8 nM and a Bmax of 1.2 pmol/mg protein were calculated.

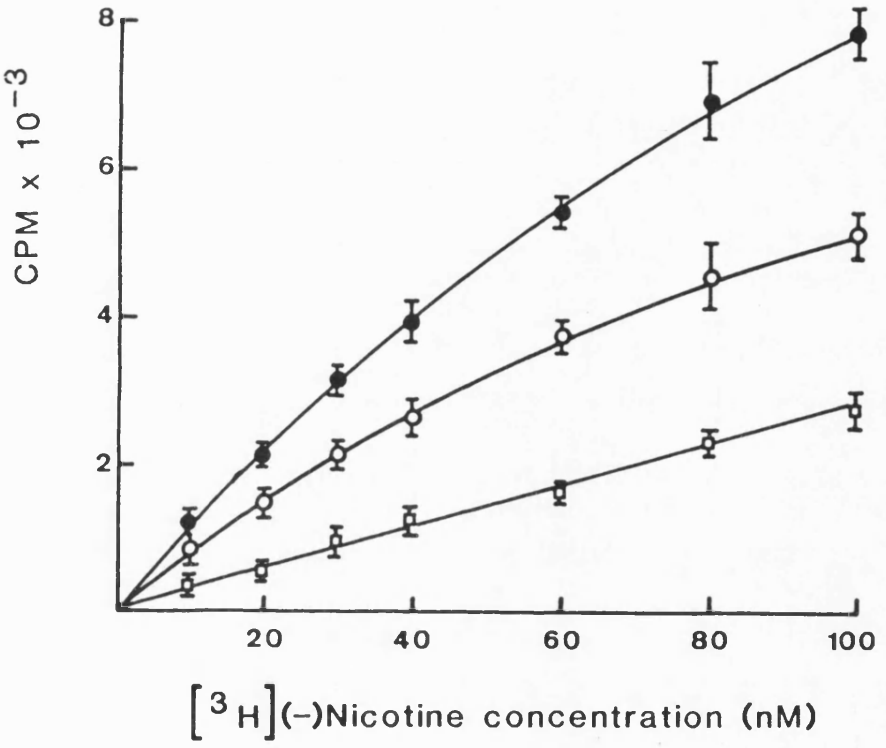


Figure 16 : The dependence of binding of (^{3}H)(-)nicotine to the P2G fraction on concentration (0-100 nM). (This result is from one experiment carried out in triplicate, typical of three). At each concentration, SD < 8%.

●—● Total binding

○—○ Specific binding

□—□ Non-specific binding

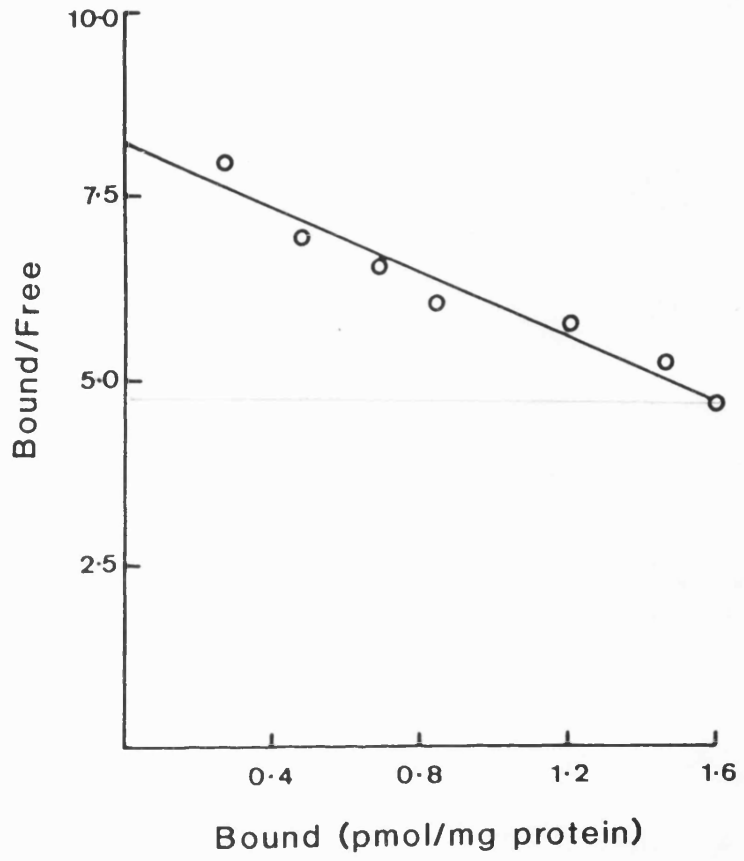


Figure 17 : Scatchard analysis of the binding data shown in figure 16. From this data a Kd of 130 nM and a Bmax of 4 pmol/mg protein was calculated.

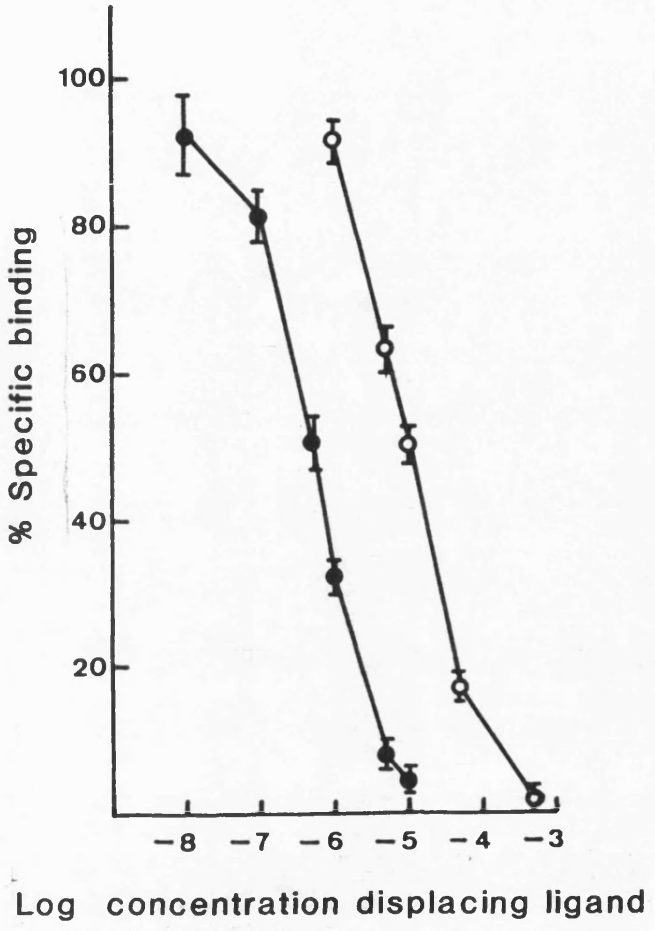


Figure 18 : Inhibition of (^{125}I) α bungarotoxin binding to the P2G fraction by (-) (●-●) and (+) (o-o) nicotine. (Results are typical of two experiments carried out in triplicate). Ki values shown in table 3, SD < 8%.

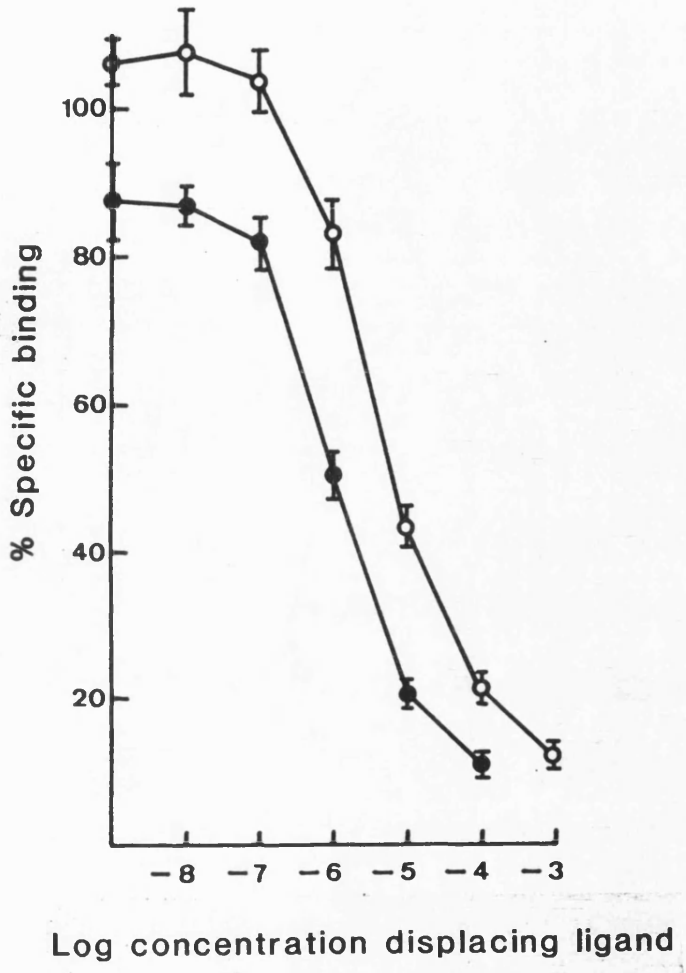


Figure 19 : Inhibition of (^{3}H)(-)nicotine binding to the P2G fraction by (-) (●-●) and (+) (o-o) nicotine. (Results are typical of three experiments carried out in triplicate). Ki values shown in table 3, SD < 8%.

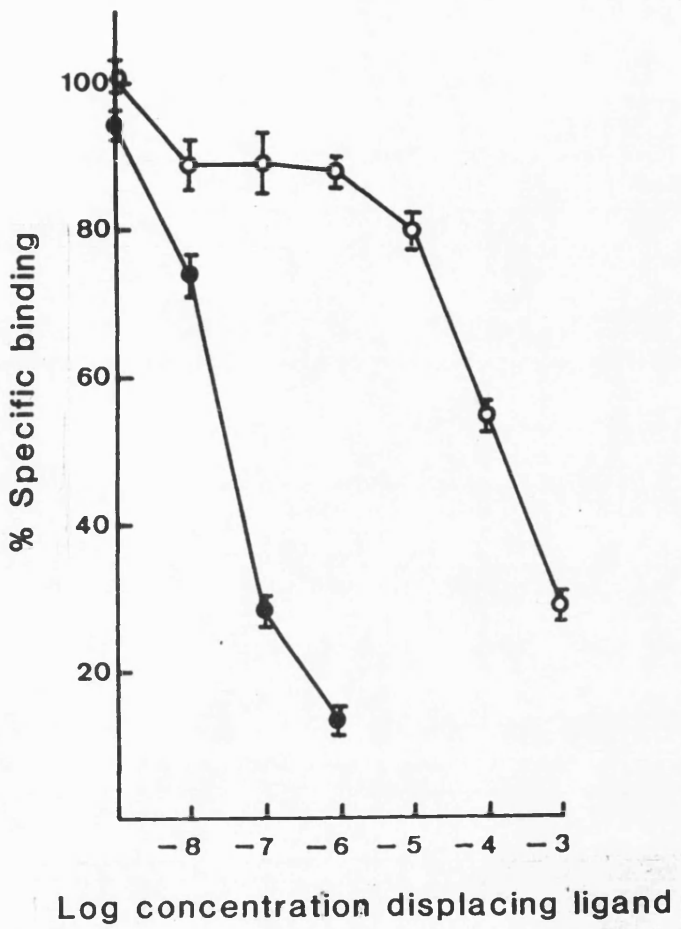


Figure 20 : Inhibition of (^{125}I) α bungarotoxin (●-●) and (^{3}H)(-)nicotine (o-o) binding to the P2G fraction by MLA. (Results are typical of three experiments carried out in triplicate). Ki values shown in table 3, SD < 8%.

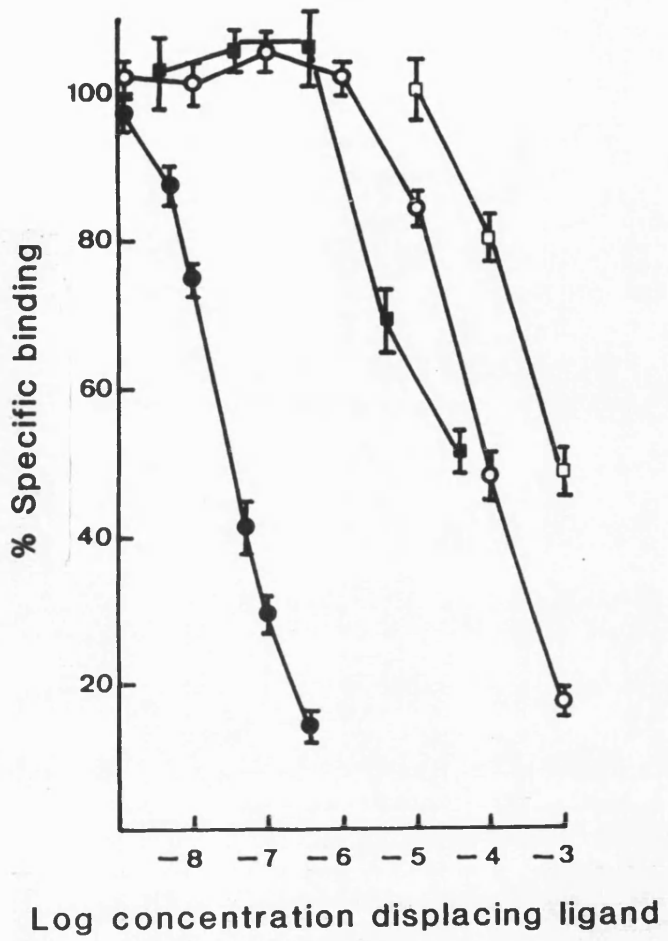


Figure 21 : Inhibition of (^{125}I) ∝ bungarotoxin (●-●; o-o) and (^{3}H)(-)nicotine (■-■ ; □-□) binding to the P2G fraction by (+) anatoxin-a (●-●; ■-■) and (-) anatoxin-a (o-o; □-□). (Results are typical of three experiments carried out in triplicate). Ki values shown in table 3, SD < 8%.

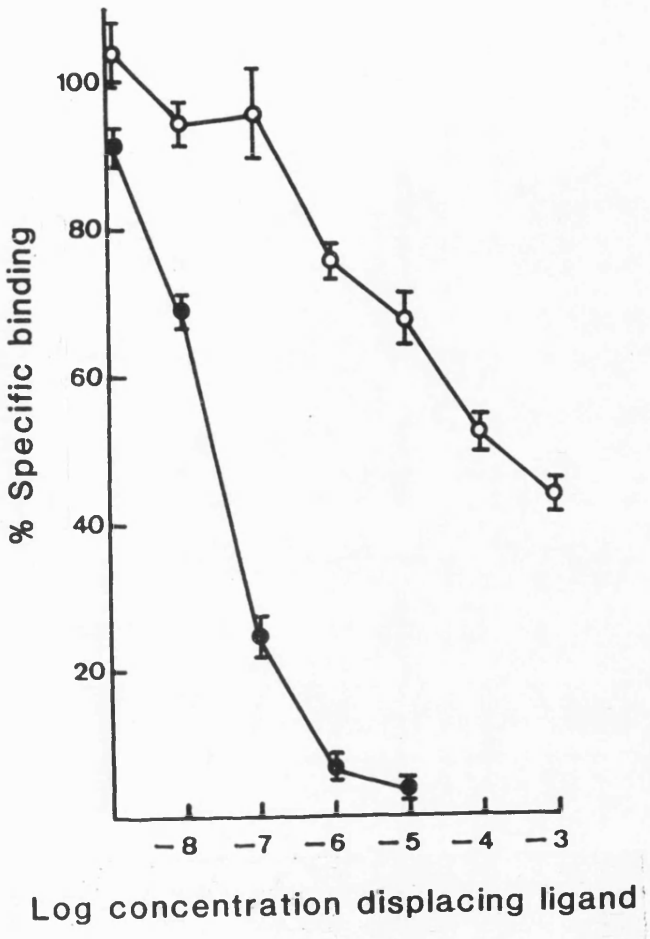


Figure 22 : Inhibition of (^{125}I) αbungarotoxin (●-●) and (^{3}H)(-)nicotine (o-o) binding to the P2G fraction by DHβE. (Results are typical of three experiments carried out in triplicate). Ki values shown in table 3, SD < 8%.

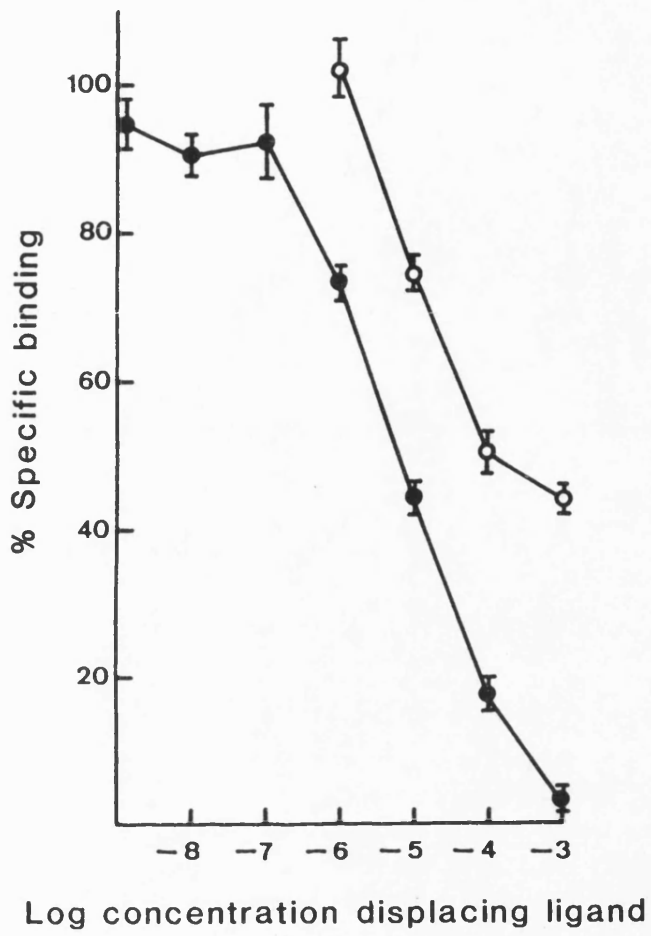


Figure 23 : Inhibition of (^{125}I) αbungarotoxin (●-●) and (^{3}H)(-)nicotine (o-o) binding to the P2G fraction by DMPP. (Results are typical of three experiments carried out in triplicate). Ki values shown in table 3, SD < 8%.

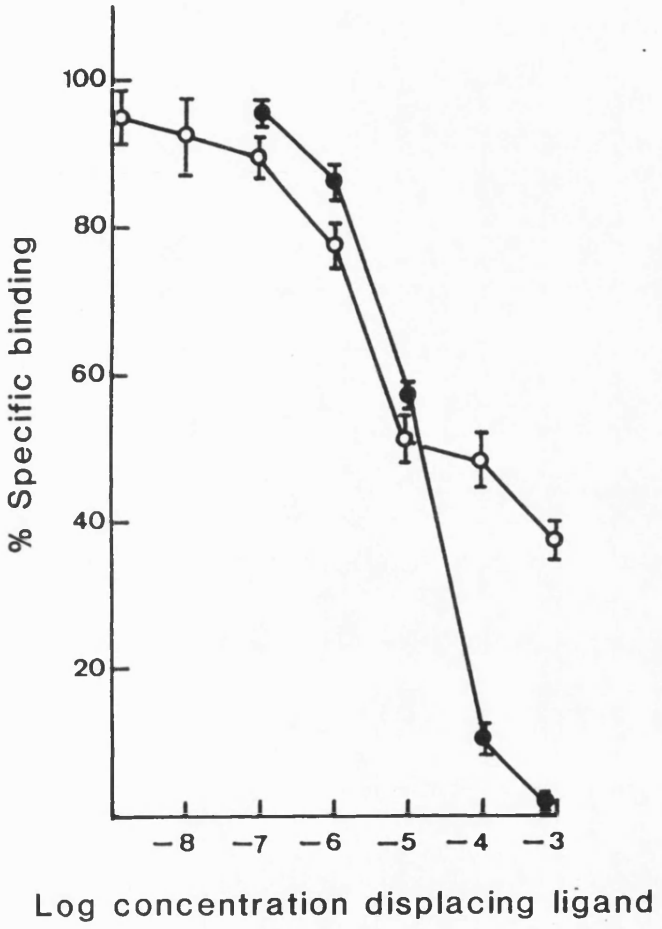


Figure 24 : Inhibition of (^{125}I) α bungarotoxin (●-●) and (^{3}H)(-)nicotine (o-o) binding to the P2G fraction by atropine. (Results are typical of two experiments carried out in triplicate). Ki values shown in table 3, SD < 8%.

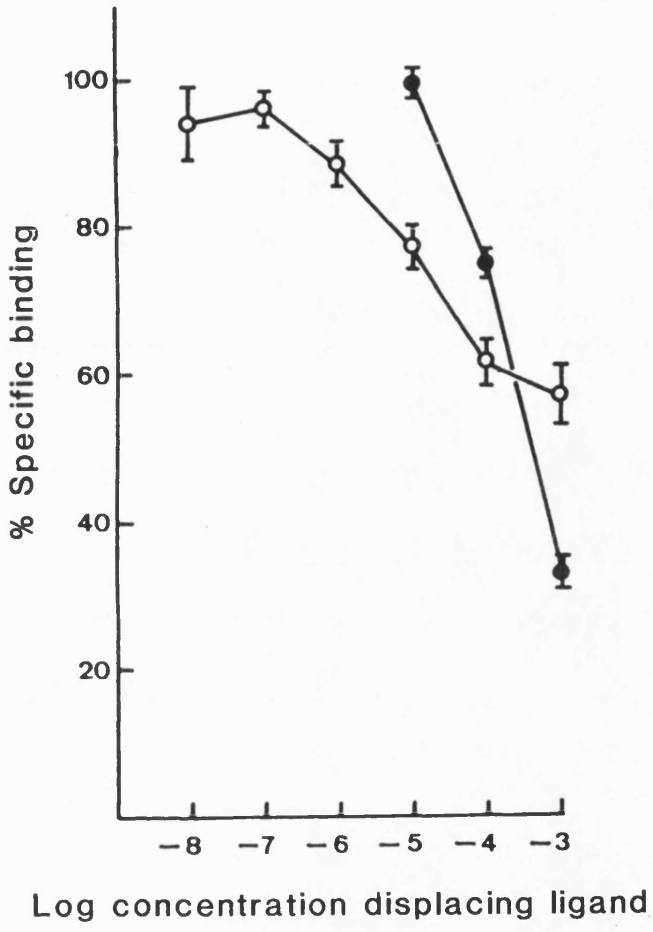


Figure 25 : Inhibition of (^{125}I) α bungarotoxin (●-●) and (^{3}H)(-)nicotine (o-o) binding to the P2G fraction by TEA. (Results are typical of two experiments carried out in triplicate). Ki values shown in table 3, SD < 8%.

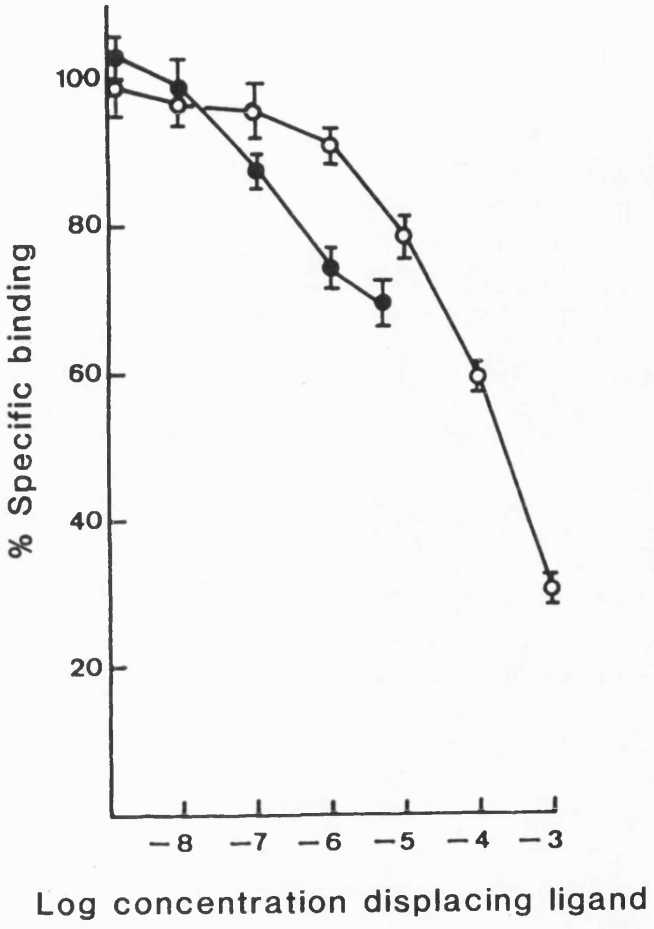


Figure 26 : Inhibition of (^{3}H)(-)nicotine binding to the P2G fraction by ∝bungarotoxin (●-●) and decamethonium (o-o). (Results are typical of two experiments carried out in triplicate). Ki values shown in table 3, SD < 8%.

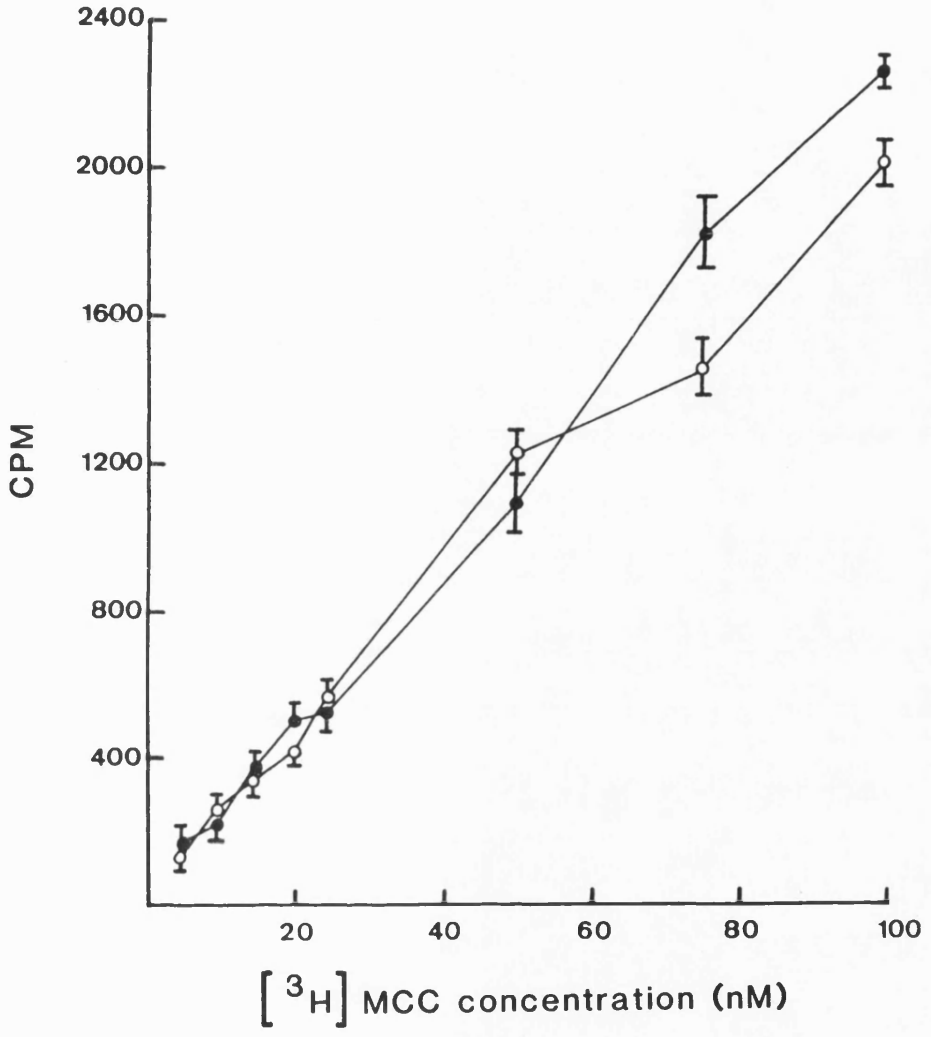


Figure 27 : The effect of increasing the concentration of (^{3}H)MCC on binding to the P2G fraction (5-100 nM). (This result is typical of two experiments carried out in triplicate). SD < 9%. Total binding ●-●; non specific binding o-o.

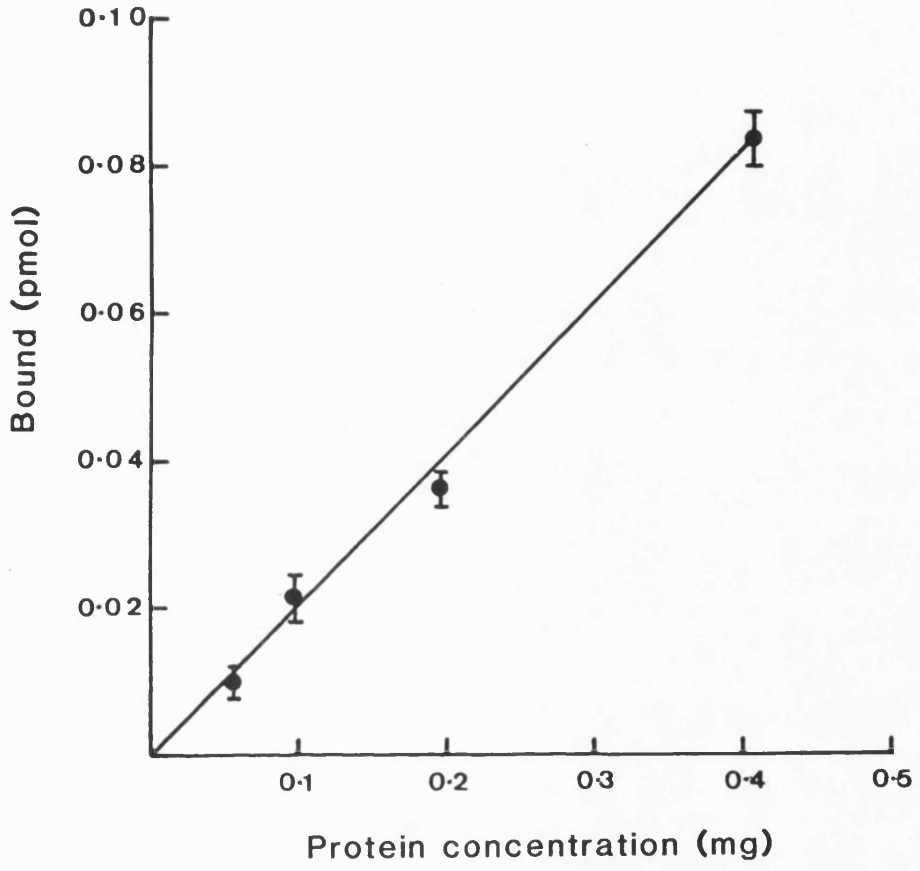


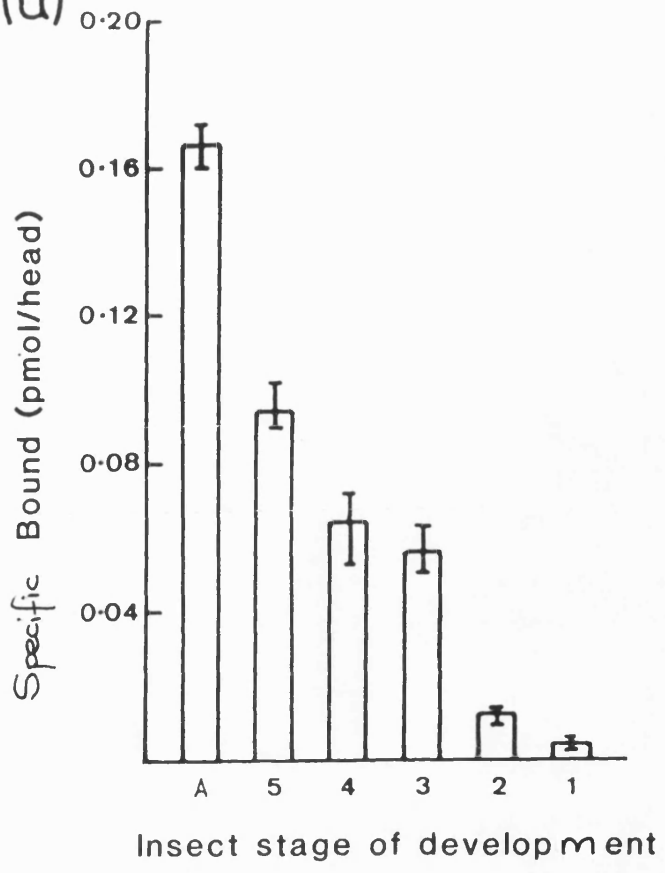
Figure 28 : Effect of varying the protein concentration on the binding of (^{125}I) αbungarotoxin to the P2H fraction prepared from adult heads. Using the same protein concentration ranges, similar graphs were obtained for the P2H fraction prepared from the heads of 1st-5th instars. (This result is from one experiment carried out in triplicate, typical of three). SD $<$ 10%.

Figure 29 : A comparison of (^{125}I) αbungarotoxin binding to the P2H fractions from adult (A) and 1st-5th instars (1-5) calculated from linearity of binding versus protein concentration experiments (e.g. see figure 28). (Results are the means of three experiments and the error bars represent the ranges).

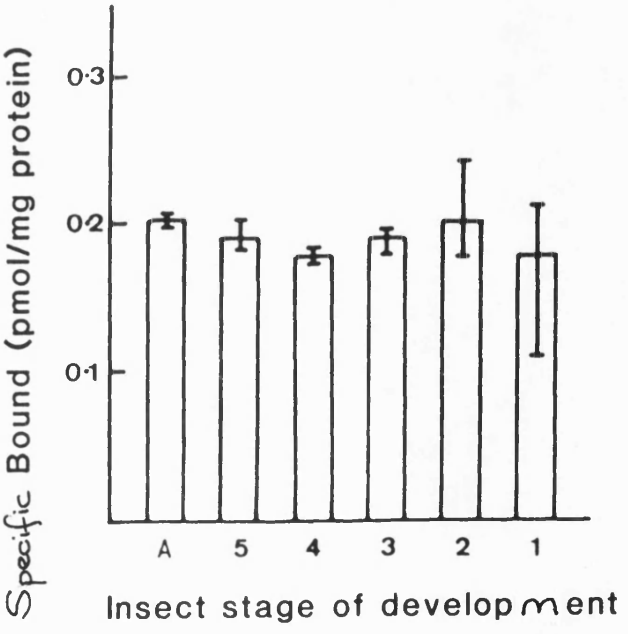
(a) Binding expressed as pmol/head

(b) Binding expressed as pmol/mg protein

(a)


0·20
0·16
0·12
0·08
0·04
Specific Bound (pmol/head)
A
5
4
3
2
1
Insect stage of development

(b)


0·3
0·2
0·1
Specific Bound (pmol/mg protein)
A
5
4
3
2
1
Insect stage of development

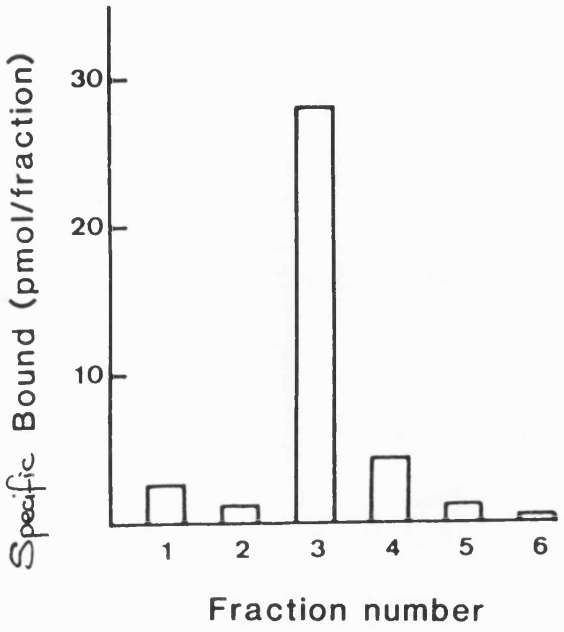


Figure 30 : Elution of the ∝bungarotoxin binding protein from the DE52 cation exchange column with buffer containing 1M NaCl. (This result is from one experiment carried out in triplicate typical of 16, see table 8). For this experiment SD < 5%.

Figure 31 : A typical silver stained denaturing gel of the α bungarotoxin binding protein purified from Schistocerca gregaria.

Lane 1 : The purified α bungarotoxin binding protein
Lane 2 : Molecular weight standards

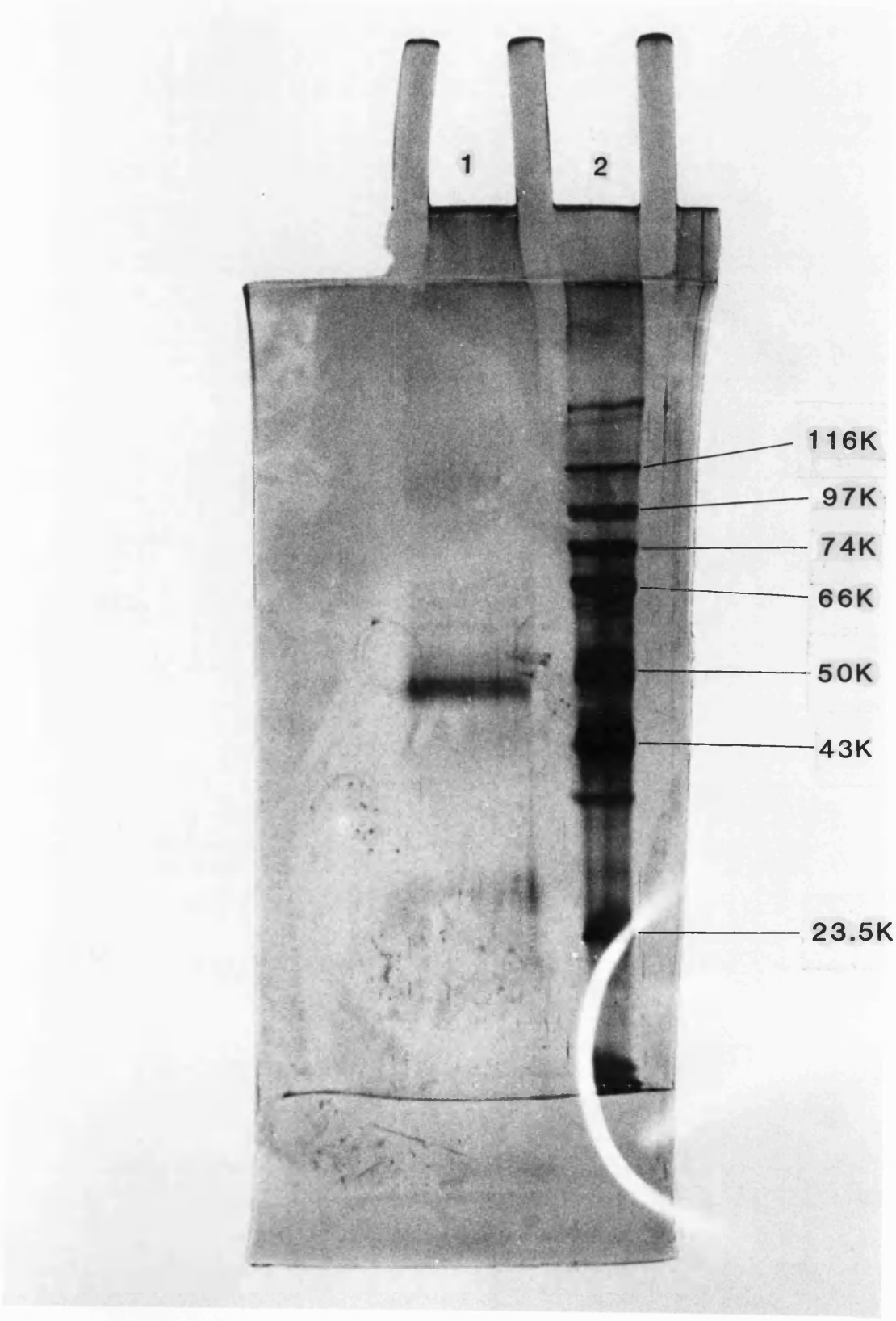

1
2
116K
97K
74K
66K
50K
43K
23.5K

Figure 32 : A silver stained denaturing gel of seven different preparations of the ∝bungarotoxin binding protein from Schistocerca gregaria showing the consistency of the 49,000 dalton band.

Lanes 2 - 8 : The purified ∝bungarotoxin binding protein

Lanes 1, 9 and 10 : Molecular weight standards

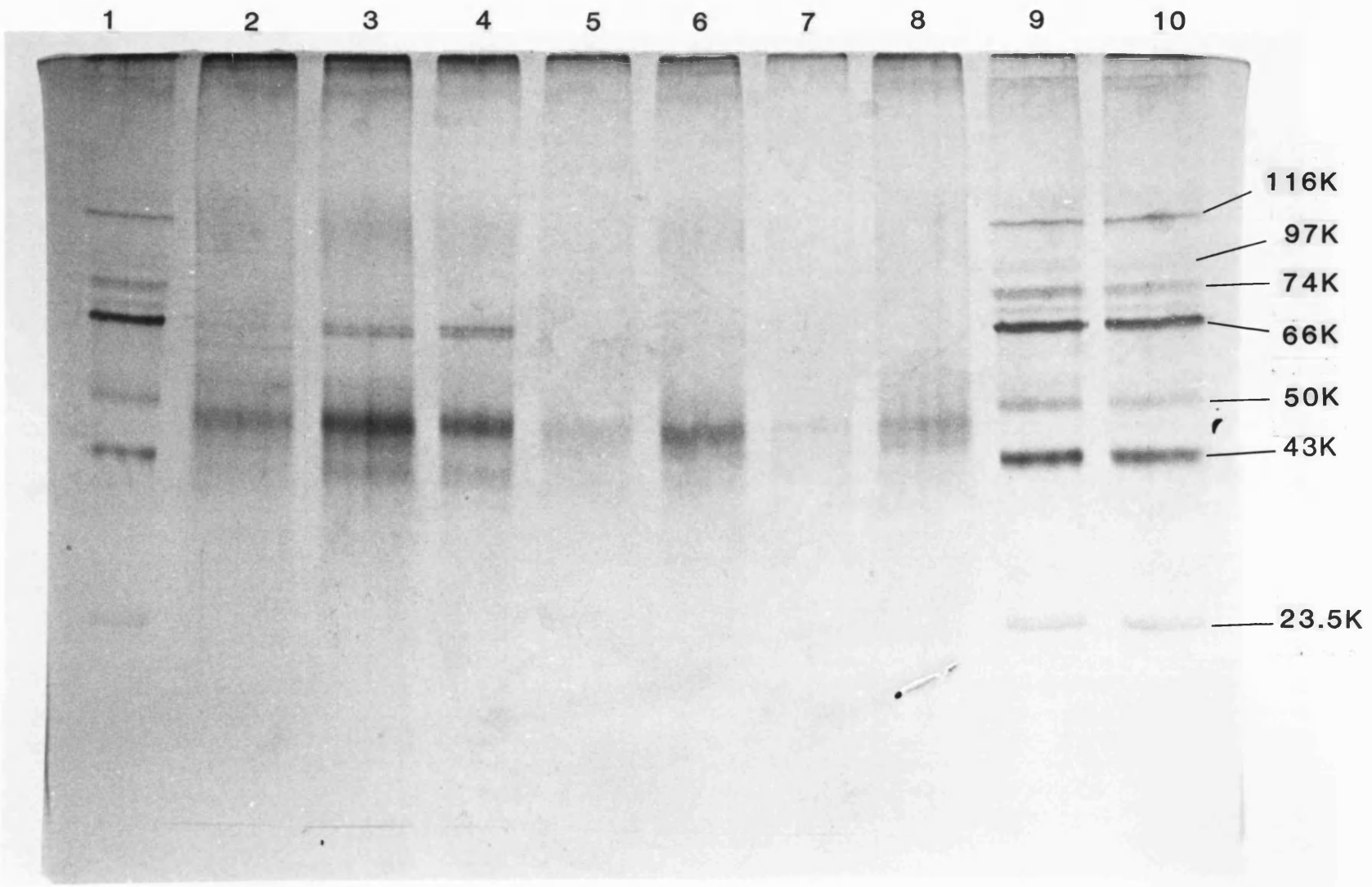

1
2
3
4
5
6
7
8
9
10
116K
97K
74K
66K
50K
43K
23.5K

Figure 33 : A comparison of the αbungarotoxin binding protein purified from Locusta migratoria and Schistocerca gregaria, analysed on a silver stained denaturing gel

Lanes 1, 3 : Schistocerca gregaria
Lane 4 : Locusta migratoria
Lanes 2, 5 : Molecular weight standards

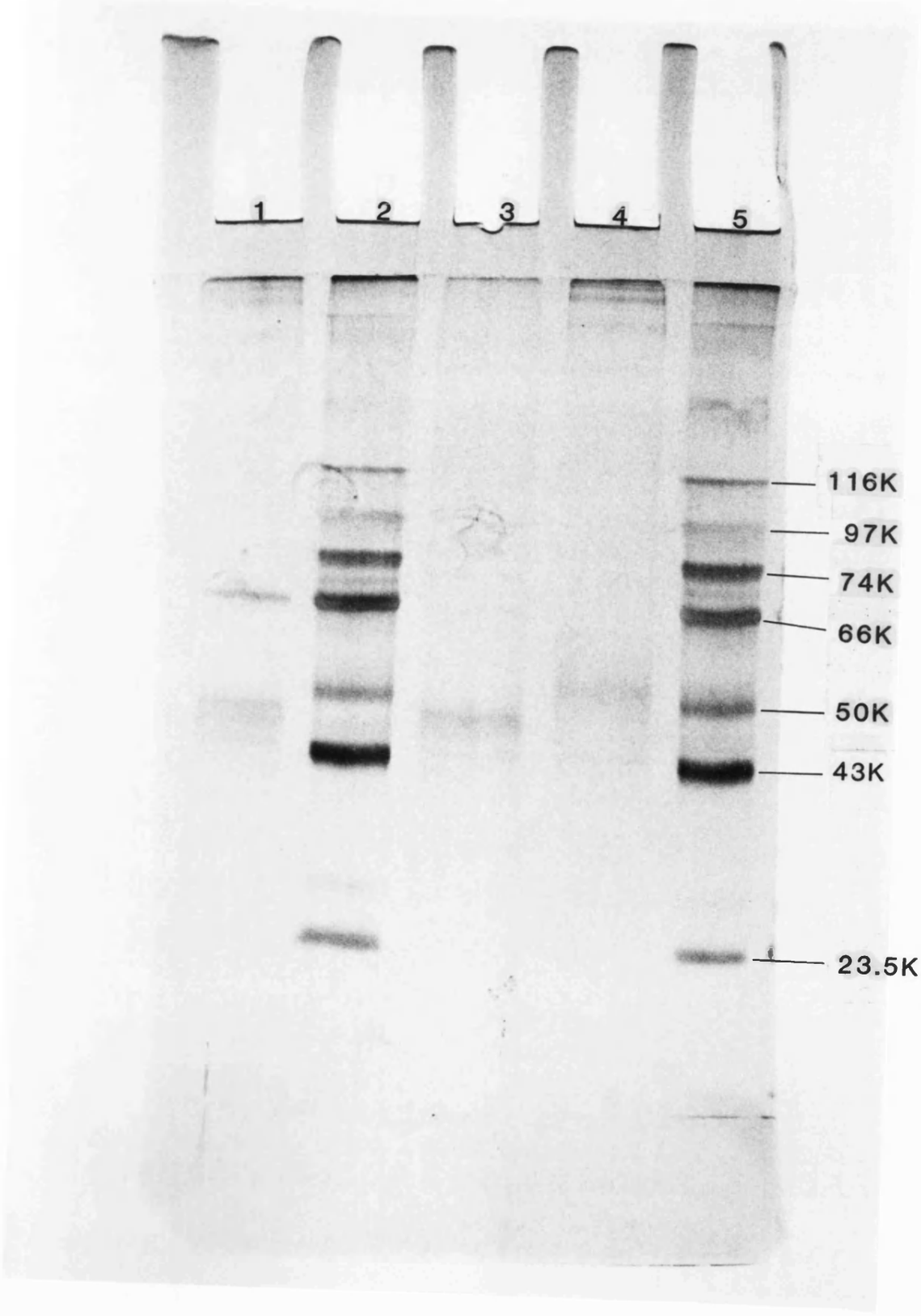

1
2
3
4
5
116K
97K
74K
66K
50K
43K
23.5K

Figure 34 : A silver stained denaturing gel of the purified αbungarotoxin binding protein from Schistocerca gregaria showing the effect of proteolytic activity in the sample.

Lane 1 : The purified αbungarotoxin binding protein which had been stored at 4°C for seven days

Lane 3 : A freshly prepared sample of the αbungarotoxin binding protein

Lanes 4,5 : Molecular weight standards

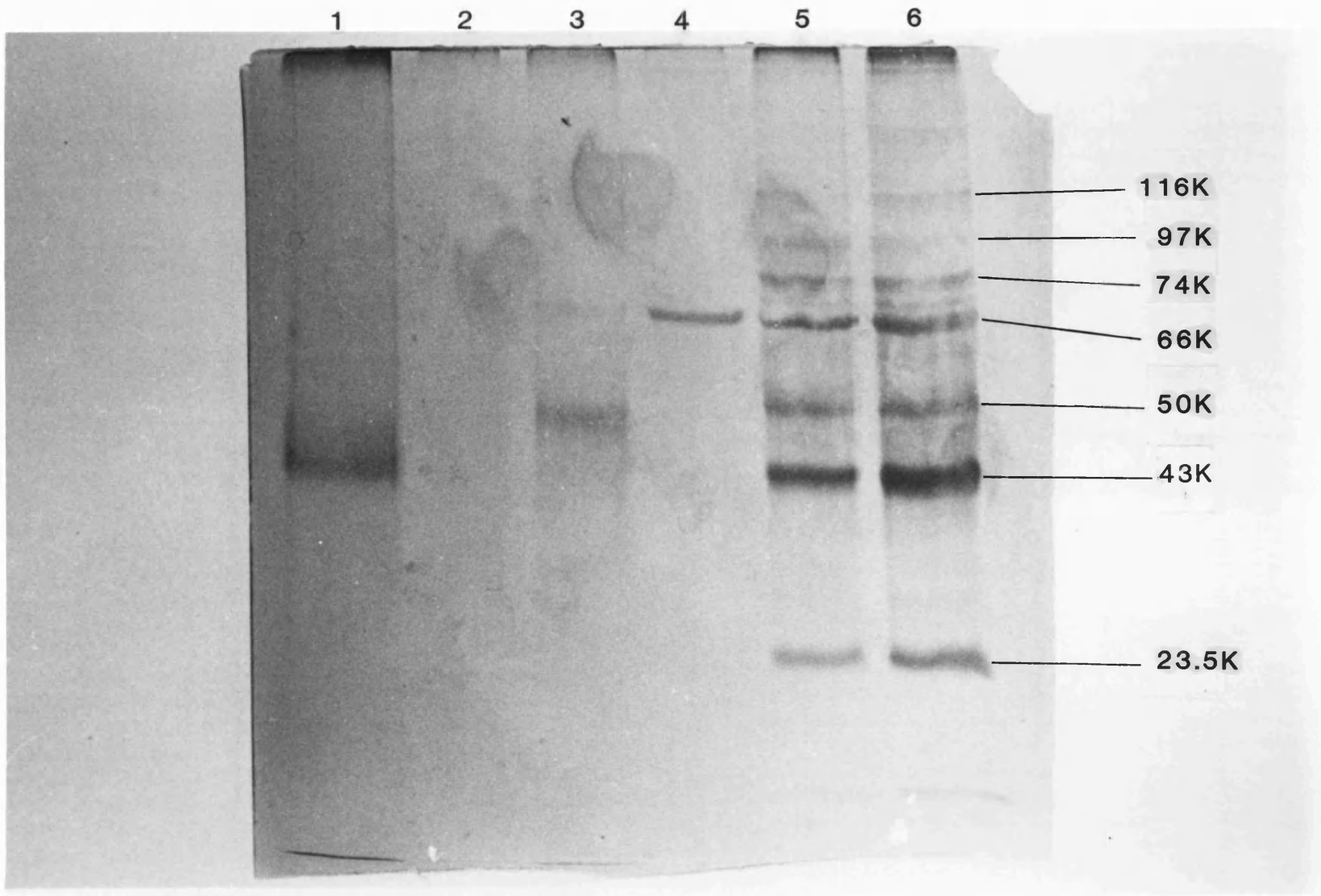
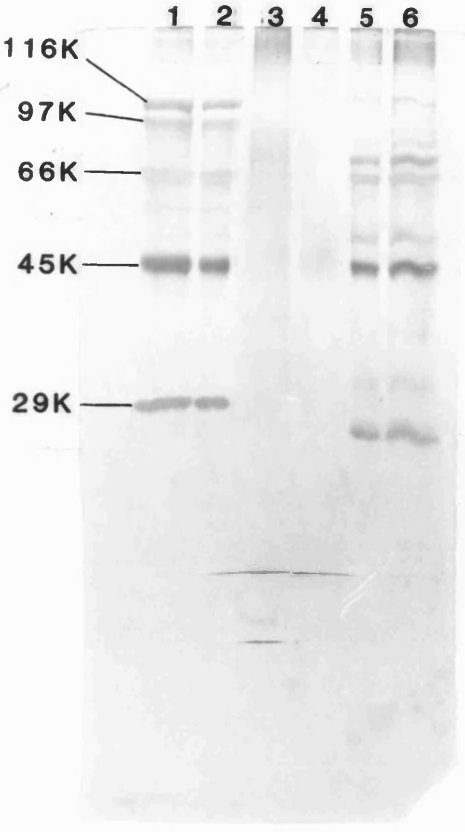
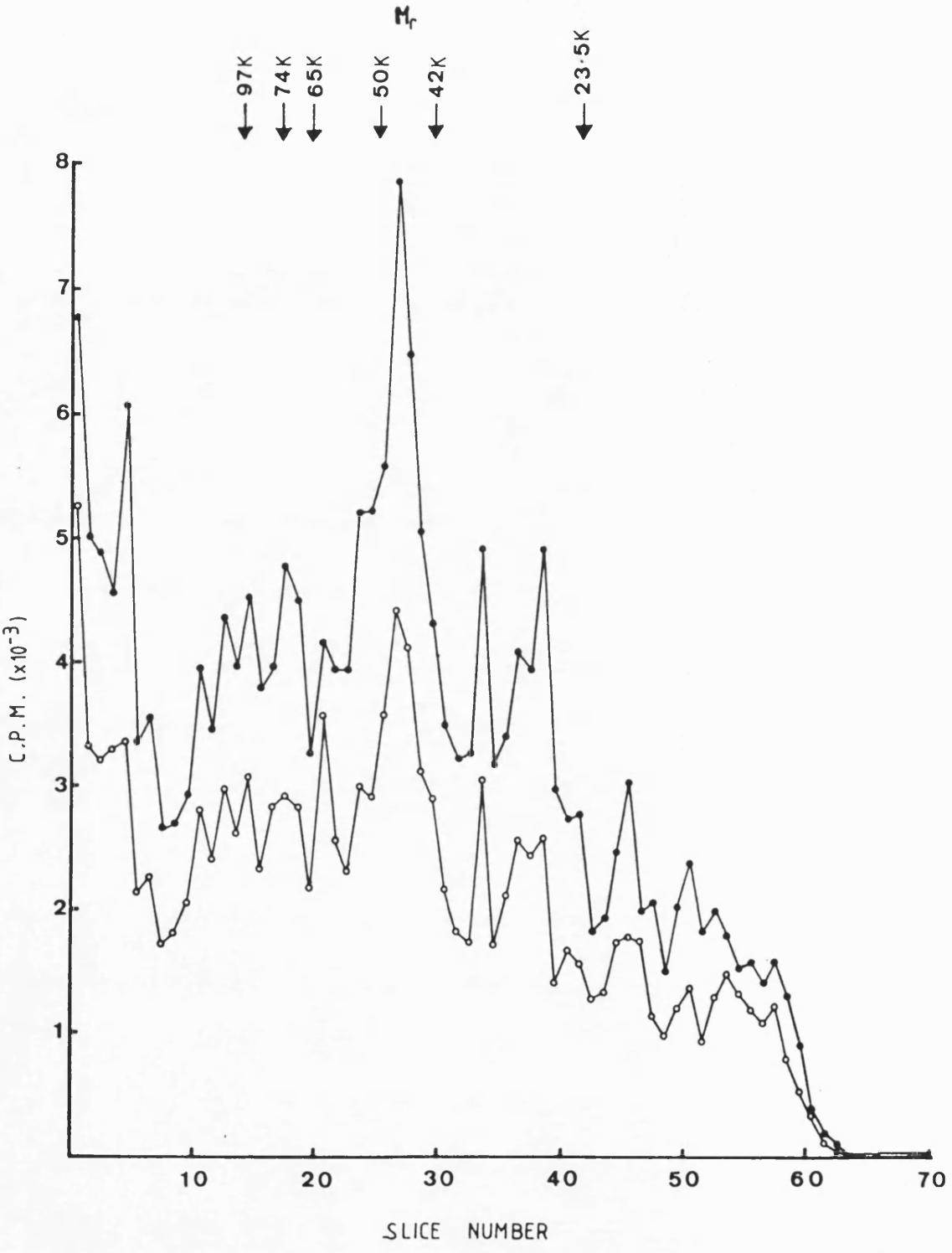

1
2
3
4
5
6
116K
97K
74K
66K
50K
43K
23.5K

Figure 35 : A silver stained denaturing gel of the purified α bungarotoxin binding protein from Schistocerca gregaria showing the effects of dialysis prior to electrophoresis.

Lanes 3, 4 : Samples of purified α bungarotoxin binding protein that had been dialysed

Lanes 1, 2, 5, 6 : Molecular weight standards


1
2
3
4
5
6
116K
97K
66K
45K
29K


M_r
97K
74K
65K
50K
42K
23·5K
C.P.M. (x10⁻³)
SLICE NUMBER
1
2
3
4
5
6
7
8
10
20
30
40
50
60
70

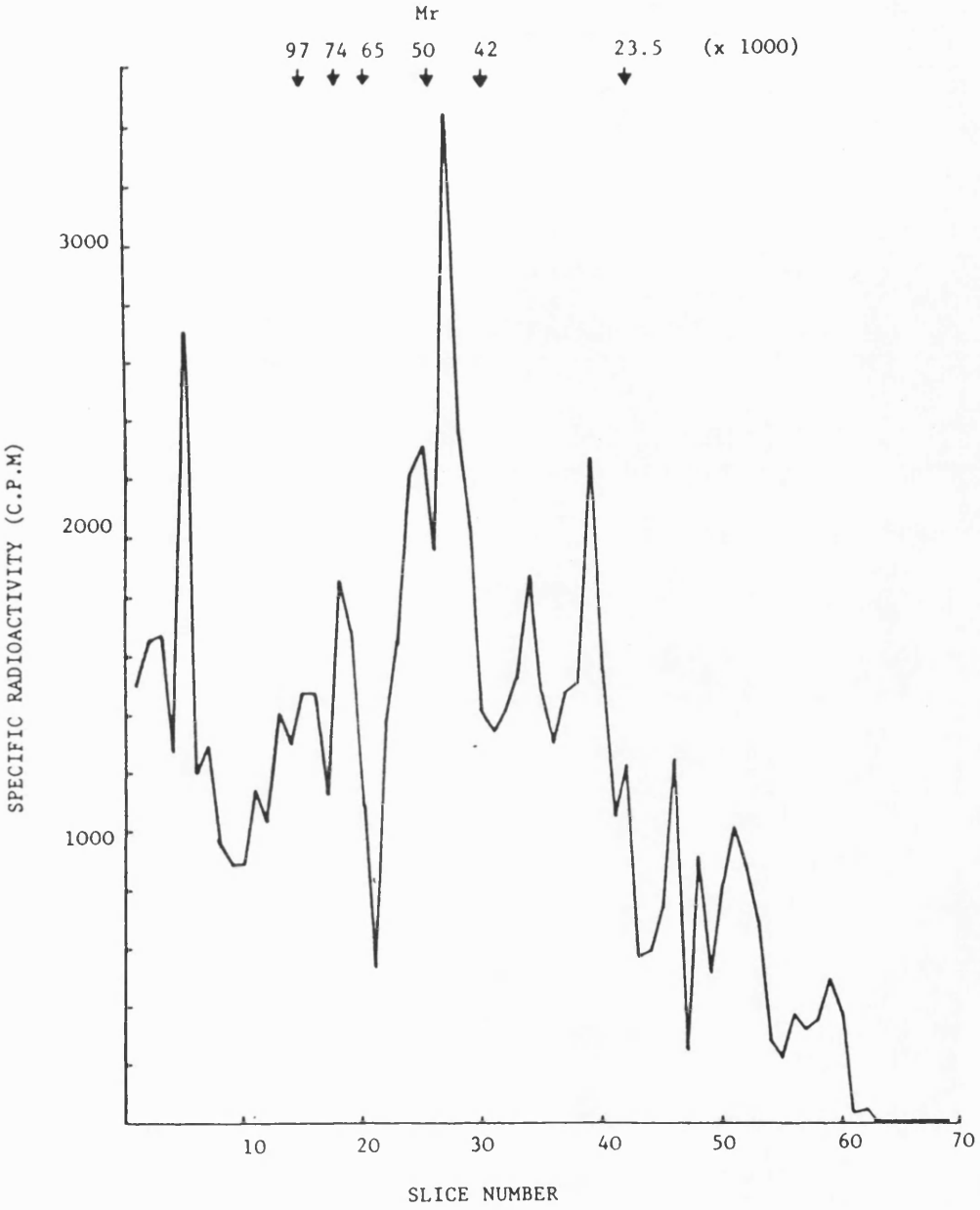


Figure 36 : Gel slice profile for the specific labelling of the P2G fraction with (^{3}H)MBTA. (This result is from one experiment typical of two).

Opposite page: Gel slice profile for the total and non-specific labelling of the P2G fraction with [^{3}H]MBTA. Non-specific labelling was determined in the presence of (-)nicotine (10^{-3}M).

●-● : Total binding. o-o : Non-specific binding.

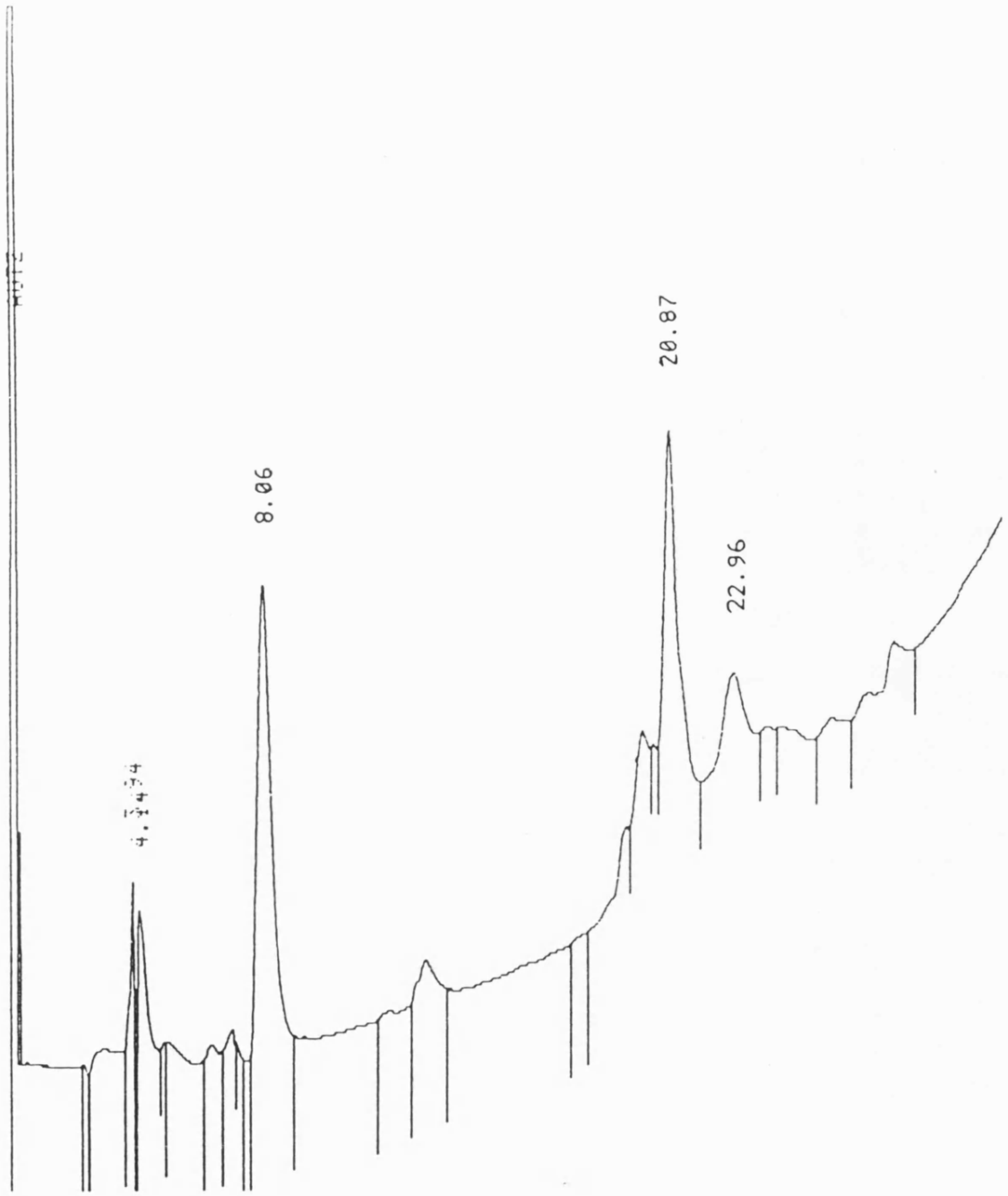


Figure 37 : HPLC analysis of the products from the initial trial cleavage of the peptide from the resin. Conditions of analysis were as described in section 2.9.2.1.

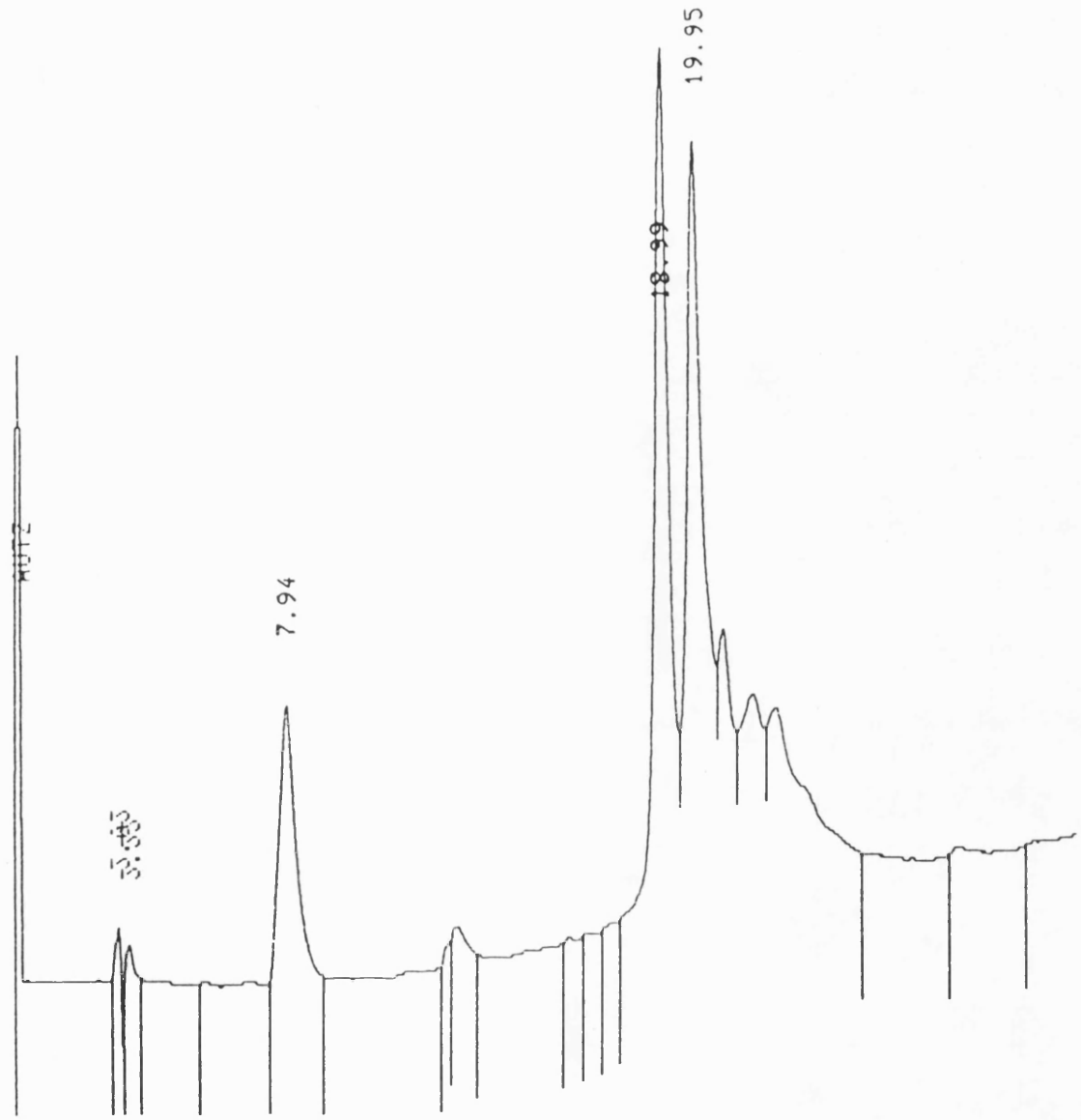


Figure 38 : HPLC analysis of the products from the second cleavage of the peptide from the resin. Conditions of analysis are described in section 2.9.2.1.

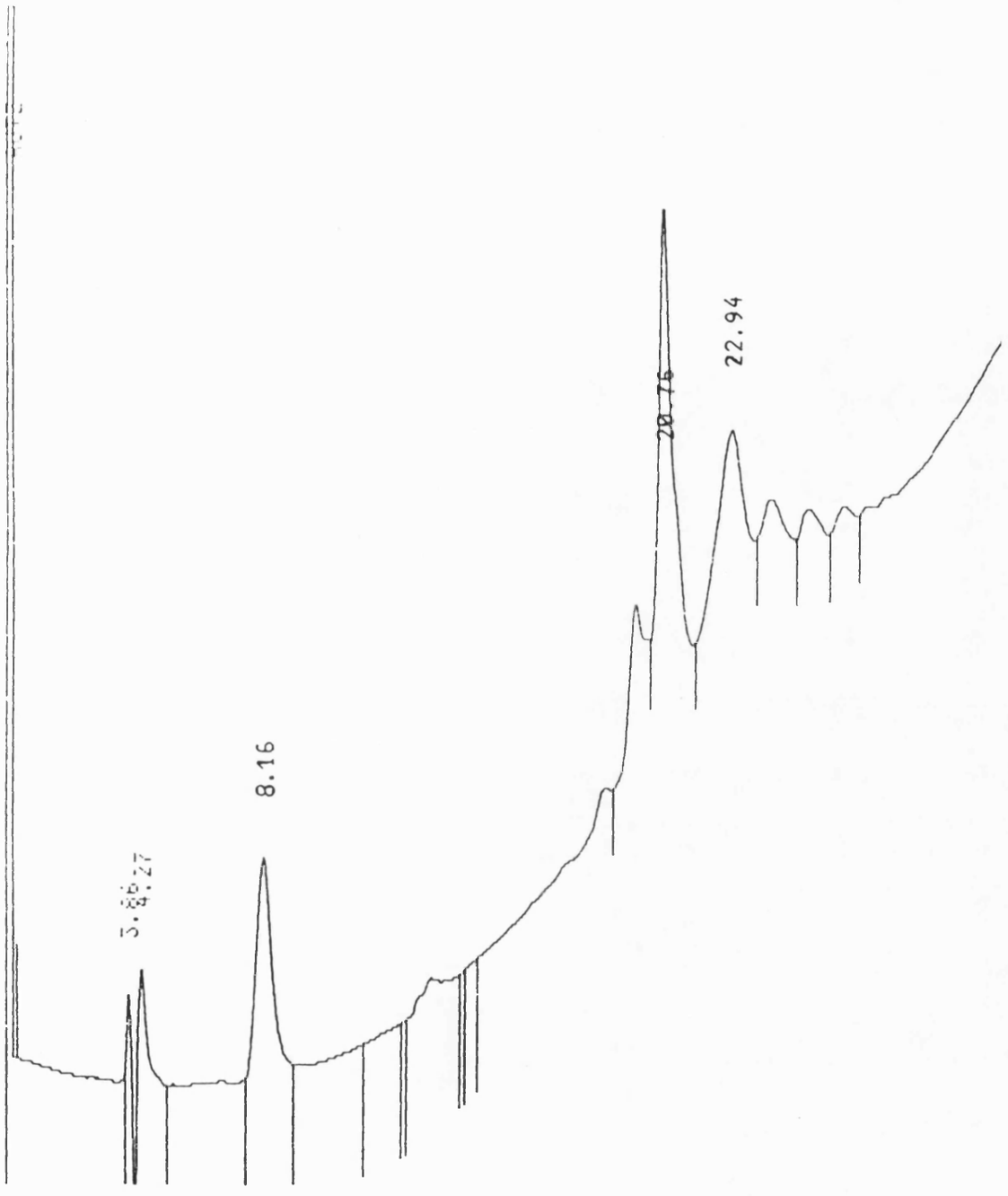


Figure 39 : HPLC analysis of the retreated peptide material from cleavage 2. Conditions of analysis are described in section 2.9.2.1.

Figure 40 : Dot blot immunobinding assay for antisera 109 and 110, raised against the synthetic peptide

Blot 1 : Preimmune serum 109
Blot 2 : Antiserum 109
Blot 3 : Preimmune serum 110
Blot 4 : Antiserum 110
Spots A : BSA (1 μg/spot)
Spots B : BSA-peptide conjugate (1 μg/spot)
Spots C : Haemoglobin-peptide conjugate (1μg/spot)
Spots D, E : Peptide alone (100 ng/spot)

All serum dilutions were at a dilution of 1/50

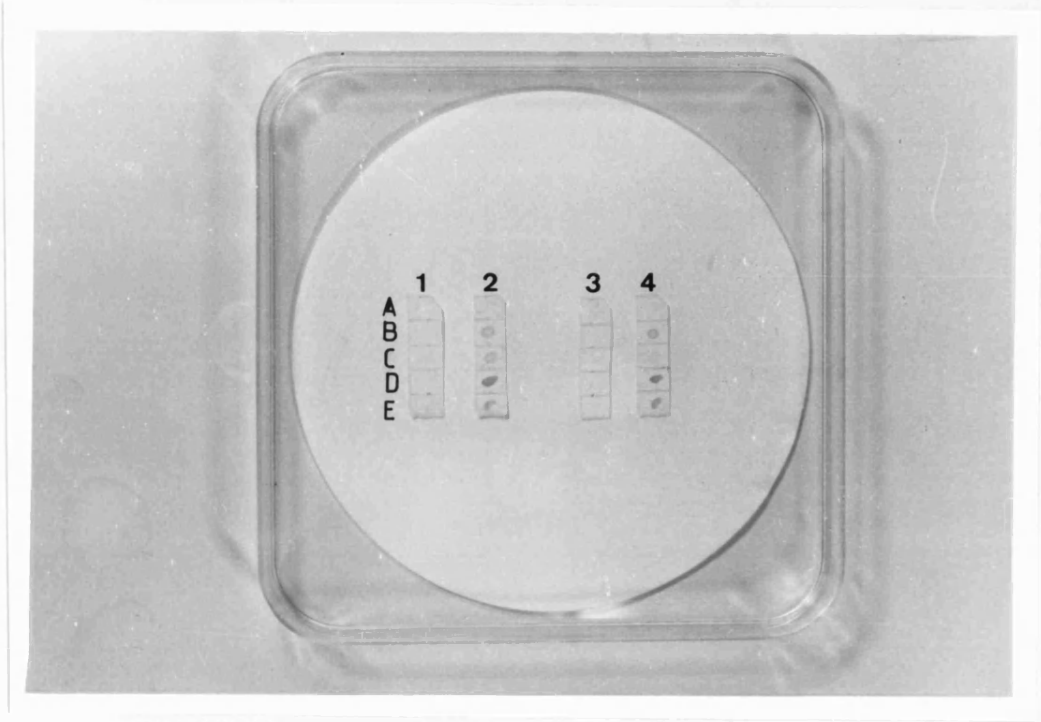

1
2
3
4
A
B
C
D
E

Figure 41 : A dot blot immunobinding assay showing the crossreactivity of antiserum Anti-L with the synthetic peptide.

Column A : Antiserum 109

Column B : Anti-L

Column C : Preimmune serum 109

Row 1 : 1/20 dilution of serums

Row 2 : 1/100 dilution of serums

Row 3 : 1/500 dilution of serums

100 ng of peptide was spotted onto each filter.

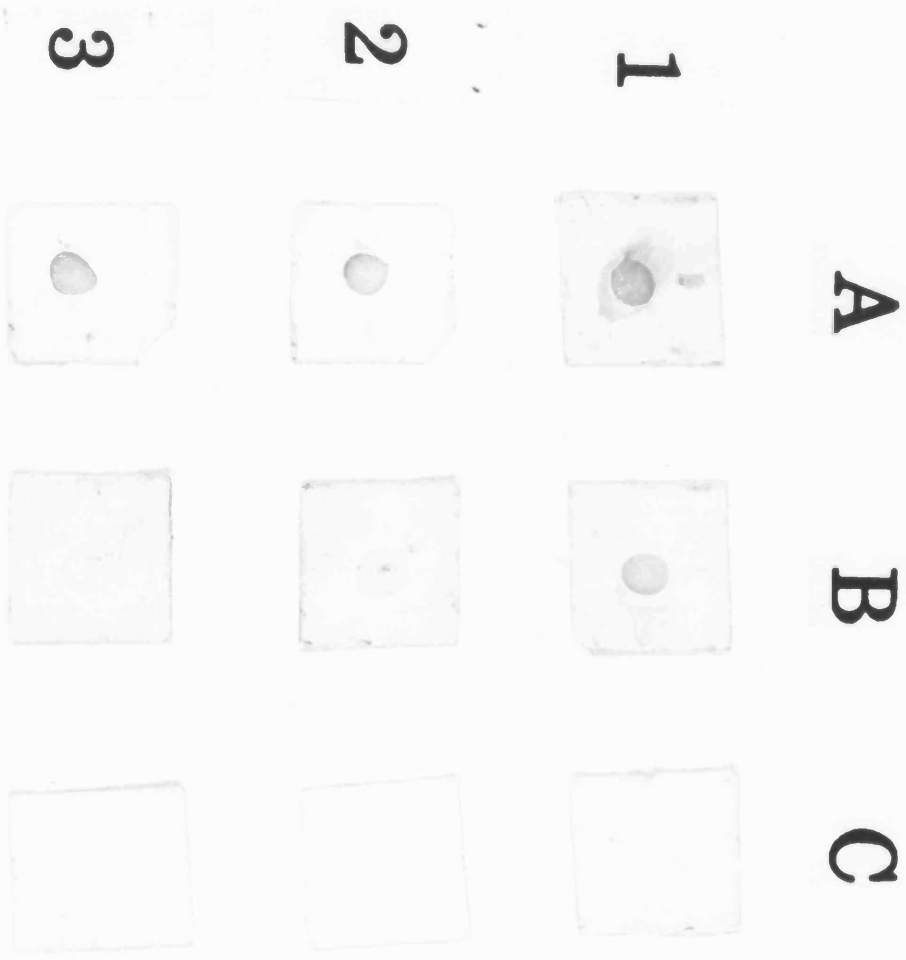

3
2
1
A
B
C

Figure 42 : Western blot of the P2G fraction analysed with AS 109

Lane 1 : AS109 1/100 dilution
Lane 2 : Preimmune serum 109 1/100 dilution

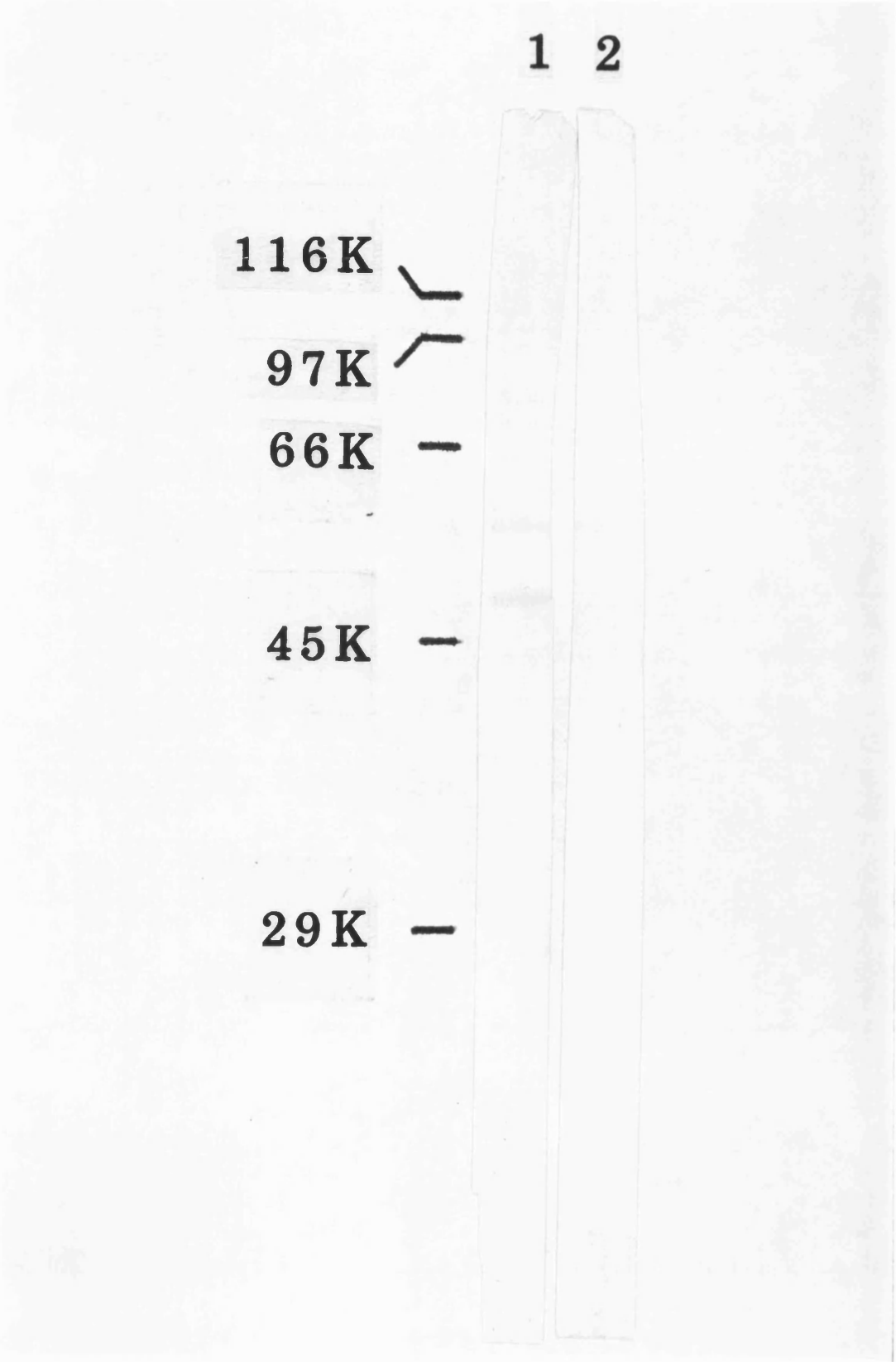
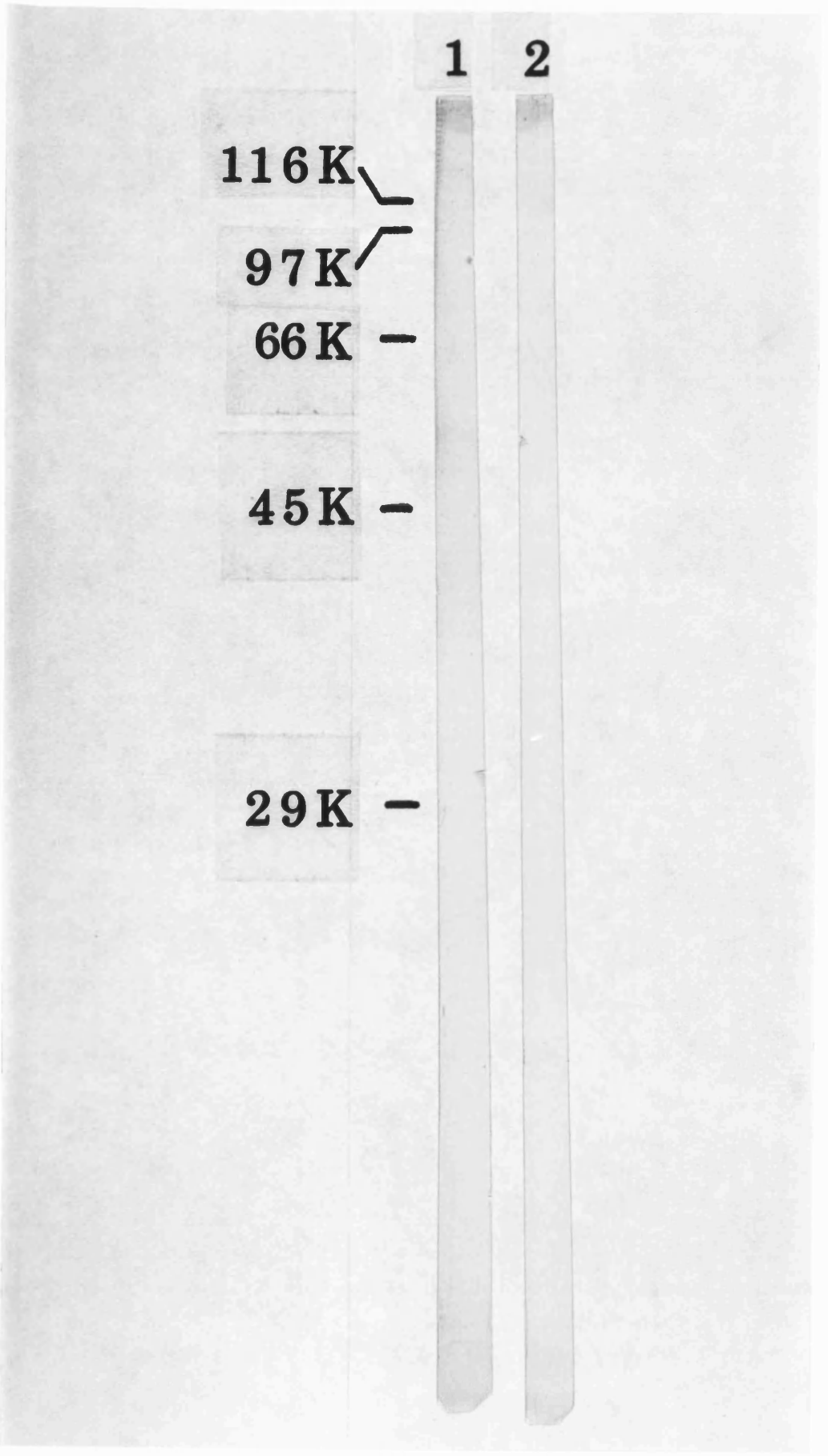

1 2
116K
97K
66K
45K
29K

Figure 43 : Western blot of the purified αbungarotoxin binding component analysed with AS 109.

Lane 1 : AS109 1/50 dilution
Lane 2 : Preimmune serum 109 1/50 dilution


1
2
116K
97K
66K
45K
29K

TABLES

Insect	Tissue	Kd (nM) (eq)	Kd (nM) (k_{-1}/k_1)	Bmax (fmol/mg)	Reference
Fruitfly	Whole head	-	-	900	Dudai, 1977
Fruitfly	Whole head	1.1	-	887	Schmidt-Nielsen et. al., 1977
Fruitfly	Whole head	1.9	0.6	800	Dudai, 1978
Fruitfly	Whole head	1.8	-	190	Rudloff, 1978
Fruitfly	Whole head	0.16	0.14	-	Jiminez and Rudloff, 1980
Housefly	Whole head	-	0.68	-	Harris et. al., 1979
Housefly	Whole head	3.1	-	204	Cattell et. al., 1980
Housefly	Whole head	3.0	-	2000	Eldefrawi and Eldefrawi, 1980
Housefly	Whole head	5.6	-	23*	Jones et. al., 1981
Locust	Cerebral ganglion	1.1	0.8	1775	Breer, 1981a
Locust	Cerebral ganglion	1.38	-	1180	Filbin et. al., 1983
Cockroach	Nerve cord	4.8	0.6	910	Lummis and Sattelle, 1985
Honeybee	Whole head	3.0	-	1000	Sherby et. al., 1986

Table 1 : A comparison of the published binding parameters for [^{125}I]αbungarotoxin binding to membrane fractions prepared from the CNS of various insects.

* Units for this value are pmol/g of heads.

Treatment	Kd (nM)	Bmax (pmol/mg)
Unwashed +	130 ± 9	4.102 ± 0.484
Buffer washed *	170	5.600
Water washed *	128	3.997

* single experiment carried out in triplicate

+ results are the average of three experiments carried out in triplicate ± S.D.

Table 2 : Comparison of the effect of washing the P2G fraction on the binding of (^{3}H)(-)nicotine. Binding was measured as described in the methods section over the concentration range for (^{3}H)(-)nicotine of 0 - 100 nM.

Ligand	Ki's (M) (^{3}H)(-)nicotine	(^{125}I) ∝bungarotoxin
(+)Anatoxin-a	$> 10^{-5}$	1.8×10^{-8}
(-)Anatoxin-a	$> 10^{-3}$	1.0×10^{-4}
(-)Nicotine	7.7×10^{-7}	5.1×10^{-7}
(+)Nicotine	4.7×10^{-6}	1.5×10^{-5}
MLA	2.5×10^{-4}	1.8×10^{-8}
DHβE	1.8×10^{-4}	1.3×10^{-8}
DMPP	2.0×10^{-4}	3.8×10^{-6}
Atropine	8.2×10^{-5}	6.3×10^{-4}
TEA	$> 10^{-3}$	1.8×10^{-4}
∝Bungarotoxin	$> 10^{-6}$	-
Decamethonium	1.4×10^{-4}	-

Table 3 : Inhibition of (^{3}H)(-)nicotine and (^{125}I) ∝bungarotoxin binding to the P2G fraction by various cholinergic ligands. Results are the average of either 2 or 3 experiments carried out in triplicate S.D. $< 8\%$. Binding assays were performed as described in the methods section with (^{3}H)(-)nicotine at a concentration of 50 nM and (^{125}I) ∝bungarotoxin at a concentration of 1 nM. Ki values were derived from IC_{50} values by the method of Cheng and Prusoff, 1973 assuming Kd values of 130 nM for (^{3}H)(-)nicotine and 1 nM for (^{125}I) ∝bungarotoxin.

Ligand	Conc^{n}	%Total Binding
(-)Nicotine	10^{-3}M	87.5
MLA	10^{-3}M	94.9
DHβE	10^{-3}M	95.9
(+)Anatoxin-a	10^{-5}M	97.3
Atropine	10^{-3}M	88.9
Carbamylcholine	10^{-3}M	99.1
TEA	10^{-3}M	91.7
α Bungarotoxin	10^{-6}M	98.5

Table 4 : Inhibition of (^{3}H)MCC binding to the P2G fraction by cholinergic ligands. Binding assays were performed as described in the methods section. (^{3}H)MCC was at a concentration of 50 nM. Results are the means of three experiments carried out in triplicate S.D. $<$ 7%.

Fraction	Total binding (pmoles)	%Recovery	Total protein content (mg)	pmoles/mg
Total homogenate	55.35	100.0	40.81	1.356
S1	0.85	1.5	9.49	0.089
P1	60.93	110.0	23.94	2.545
S2 (detergent extract)	45.28	81.8	19.52	2.320
P2	0.34	0.6	0.94	0.362

Table 5 : Distribution of (^{125}I) αbungarotoxin binding activity in the solubilisation procedure. Binding assays were performed as detailed in the methods section with (^{125}I) αbungarotoxin at a concentration of 10 nM. Results are the means of three experiments carried out in triplicate S.D. < 15 %

Fraction	Total binding (pmoles)	%Recovery	Total protein content (mg)	pmoles/mg
P1 membranes	65.61	100.0	25.32	2.591
0.1% Lubrol PX	29.88	45.5	17.67	1.691
1% Lubrol PX	50.52	77.0	20.01	2.525
3% Lubrol PX	51.96	79.2	20.26	2.565
5% Lubrol PX	50.40	76.8	20.74	2.430
10% Lubrol PX	41.16	62.7	21.24	1.938

Table 6 : Effect on the solubilisation of the (^{125}I) α bungarotoxin binding component by varying the detergent concentration. (^{125}I) α bungarotoxin was at a concentration of 10 nM. Binding assays were performed as described in the methods section. Results are the means of three experiments carried out in triplicate S.D. $<$ 8%.

(^{3}H)(-)Nicotine concentration (nM)	pmoles bound / mg
40	0.008
60	0.006
100	0.030

Table 7 : (^{3}H)(-)Nicotine binding to the detergent extract. Binding assays were performed as detailed in the methods section. Results are the mean of two independent experiments carried out in triplicate S.D. $<$ 6%.

Purification	pmoles/100 ganglia	protein weight equivalent (μg)
1	17.7	0.867
2	28.0	1.372
3	6.2	0.304
4	15.4	0.755
5	9.6	0.470
6	23.0	1.127
7	22.4	1.098
8	24.6	1.205
9	18.4	0.902
10	31.6	1.548
11	12.5	0.613
12	10.5	0.515
13	14.6	0.715
14	11.8	0.578
15	27.4	1.343
16	32.1	1.573

Table 8 : A comparison of the quantity of (^{125}I) α bungarotoxin binding activity purified from different preparations. Protein weight equivalents were calculated assuming a 1:1 binding of (^{125}I) α bungarotoxin to the 49,000 Mr polypeptide. Binding assays were performed as detailed in the methods section with (^{125}I) α bungarotoxin at a concentration of 10 nM. Each binding experiment was carried out in triplicate.

Amino acid	%Area / %Area for Lysine				
	Peak 2	Peak 4	Peak 5	Peak 6	Expected
Asp	2.157	2.070	2.124	2.089	2
Thr	3.222	2.687	2.873	2.971	3
Ser	2.134	1.684	1.890	2.013	2
Gln	3.503	3.190	3.241	3.382	3
Cys	0.501	0.703	0.818	0.928	2
Val	0.972	0.926	1.205	0.963	1
Met	0.842	0.946	0.944	0.958	1
Ile	1.818	1.936	1.913	1.949	2
Tyr	0.811	0.203	0.344	0.785	1
Phe	2.295	2.419	2.318	2.352	2
Lys	1.000	1.000	1.000	1.000	1

Table 9 : Amino acid analysis of peptides identified by HPLC. The peak area for each amino acid was determined from the analysis trace and the percentage area was related to that for lysine, since lysine was the first amino acid in the sequence. Proline was also present in all the peptide fractions analysed, detected at 440 nM instead of 570 nM. The values for cysteine are lower than expected, possibly due to the fact that the protecting group was not removed by the cleavage process.

Antibody	% of (^{125}I) α bungarotoxin binding activity remaining
Anti-L	43.0
Anti-T	77.2
Mab 270	100.1
Mab 290	98.3
Mab 299	99.0
Mab B11	100.0
Mab C07	101.0
Mab C11	98.0
Mab E08	96.6
As 109	100.2

Table 10 : Immunodepletion studies. These binding experiments were performed as detailed in the methods section. Results are the average of three experiments carried out in triplicate S.D. $<$ 6%.

Sample addition	Volume (μl)	(^{125}I) α bungarotoxin bound (CPM)	% Inhibition
Buffer	10	574226	-
αBungarotoxin	10(10^{-5}M)	76676	86.6
Preimmune	10	467152	-
As 109	10	461337	1.2
Preimmune	5	498864	-
As 109	5	488327	2.1
Preimmune	2	497291	-
As 109	2	489706	1.5

Table 11 : Lack of inhibition of (^{125}I) α bungarotoxin binding to the detergent extract by As 109. The assay of (^{125}I) α bungarotoxin binding to the detergent extract was performed as detailed in the methods section. Sample additions were incubated for 60 minutes at 20^{o}C prior to addition of (^{125}I)αbungarotoxin (final concentration of 10 nM). Preimmune serum was used as the control binding from which the inhibition by As 109 could be calculated. Results are from a single experiment carried out in triplicate.

Sample addition	Volume (μl)	(^{125}I) α bungarotoxin bound (CPM)	% Inhibition
Buffer	10	51069	-
αBungarotoxin	10(10^{-5}M)	6973	86.4
Preimmune	10	48216	-
As 109	10	47829	0.8
Preimmune	5	49351	-
As 109	5	49184	0.3

Table 12 : Lack of inhibition of (^{125}I) α bungarotoxin binding to the P2G fraction by As 109. The assay of (^{125}I) α bungarotoxin binding to the P2G fraction was performed as detailed in the methods section. Sample additions were incubated for 60 minutes at 20^{o}C prior to addition of (^{125}I) α bungarotoxin (final concentration of 10 nM). Preimmune serum was used to determine control binding from which the inhibition by As 109 could be calculated. Results are from a single experiment carried out in triplicate.

Experiment	Injection	(^{125}I) αbungarotoxin bound (pmol / oocyte)
1	locust head mRNA	0.178
	locust ganglia mRNA	0.131
2	locust ganglia mRNA	0.020
3	locust ganglia mRNA	0.243
4	Torpedo mRNA	0.233

Table 13 : Assay of specific binding to detergent extract of oocytes injected with mRNA. Control uninjected oocytes were assayed alongside in each experiment and no binding was detected. Binding was assayed as detailed in the methods section with (^{125}I) α bungarotoxin at a concentration of 10 nM.

	(^3H)(-)Nicotine		(^{125}I) ∝ Bungarotoxin	
	Locust ganglia	Rat Brain	Locust ganglia	Rat Brain
Kd (nM)	130	9	0.8	1.5
Bmax (pmoles/ mg protein)	4	0.1	1.2	0.06

Table 14 : Comparison of the binding characteristics of (^3H)(-)nicotine and (^{125}I) ∝ Bungarotoxin binding to the locust P2G fraction and a rat brain P2 membrane fraction. (Reproduced from MacAllan et. al., 1988).

RANK ORDER OF POTENCY OF LIGANDS

(^{3}H)(-)Nicotine			(^{125}I) ∝ Bungarotoxin	
Rat Brain	Locust ganglia		Rat Brain	Locust ganglia
(+)ANA	(-)NIC	HIGHEST	MLA	DHβE
(-)NIC	(+)NIC	↑	(+)ANA	MLA *
(-)ANA	(+)ANA *		(-)NIC	(+)ANA
DHβE	DHβE		(-)ANA	(-)NIC
MLA	MLA		DHβE	(+)NIC
(+)NIC	(-)ANA	LOWEST	(+)NIC	(-)ANA

TABLE 15 : A comparison of the rank order of potency of several cholinergic ligands inhibiting (^{3}H) (-)Nicotine and (^{125}I) ∝ bungarotoxin binding to the locust P2G fraction and rat brain P2 membranes. (Rat brain data taken from MacAllan et. al., 1988).

* Boxes represent ligands with essentially the same potency

CHAPTER 4

DISCUSSION

The work reported here has focussed on attempts to further characterise the putative nAChR of insect ganglia. Previously published work on this receptor has relied almost entirely on the use of ∝bungarotoxin, therefore a comparison was made of the binding of (^{125}I) ∝ bungarotoxin, (^{3}H)(-)nicotine and (^{3}H)MCC to a locust P2G membrane fraction. These results will be compared to a parallel study carried out on rat brain P2 membranes by Dr Sue Wonnacott (MacAllan et. al., 1988) and the significance of the insect binding sites will be discussed. Attempts at improved purification and characterisation of the bungarotoxin binding protein have also been made in this study and these results are compared with those from other insects. Finally, this discussion will describe early results in the application of molecular biology to the identification of the insect nAChR and the future direction in this field.

4.1 Criteria for the Identification of Neurotransmitter Receptors

Characterisation of neurotransmitter receptor proteins usually starts with the identification of specific binding sites for a radiolabelled ligand. However, this alone is not sufficient to classify the binding site as a physiological receptor and further characterisation must be made. The criteria for the identification of a physiological neurotransmitter receptor are as follows :-

1) Identification of a specific binding component for the transmitter or a well characterised agonist or antagonist which saturates with increasing concentrations;

2) Specific binding should increase linearly with protein concentration;

3) Specific binding should be displaced by pharmacologically related ligands at physiological concentrations but not by ligands of different pharmacological specificity.

4) Specific binding sites must be localised to areas known to show physiological responses to the ligand.

5) Specific binding sites should be isolated, purified and characterised biochemically.

6) The isolated specific binding protein should be reconstituted into an artificial lipid bilayer and be shown to have the functional properties of the original site.

The nAChR from the Torpedo electric organ has been shown to satisfy all these criteria. There are also reports in

the literature dealing with each of these areas for the putative insect nAChR (see introduction 1.11). However, there is still much work to be done on the insect nAChR to fully characterise this protein. The results in this thesis go some way towards a better understanding of the pharmacology and structure of the insect nAChR and more specifically that found in the ganglia of Schistocerca gregaria.

4.2 A Comparison of Cholinergic Binding Sites in Insects

In this study, the binding of (^{125}I) α bungarotoxin to a locust ganglionic P2 membrane fraction was compared with that for (^{3}H)(-)nicotine. This work was inspired by current work on vertebrate brain nAChR's that are insensitive to α bungarotoxin but which bind (^{3}H)(-) nicotine with high affinity (see Wonnacott, 1987). The type of binding assay developed for this study was therefore based on that which had previously been used for the vertebrate nervous system. (^{3}H)(-)Nicotine binding to vertebrate neural tissue is found to have a fast rate of dissociation and therefore requires a method a rapid separation of the free radioligand from that bound to the membranes. This is usually achieved by filtration of the samples on glass fibre filters such as Whatman GFC filters. A modification of this filtration method was adopted for the binding assays reported here, which arose from the work on the purified α bungarotoxin binding

protein in this study. When glass fibre filters are soaked in a solution of 0.3% PEI they are converted from a negative charge to a positive charge and can therefore be used in the same way as DE81 cellulose discs for the assay of solubilised receptors (Bruns et. al., 1983). A comparison of DE81 discs with PE1 treated GFB filters revealed that the PE1 treatment also had the effect of reducing background radioactivity for the assay of (^{125}I) α bungarotoxin binding to the purified α bungarotoxin binding component. A similar reduction in background was also observed in assays of (^{3}H)(-)nicotine binding to rat brain P2 membranes (Wonnacott, personal communication). Therefore this filtration method was used for both the (^{125}I) α bungarotoxin and (^{3}H)(-)nicotine binding assays with the locust ganglionic P2 fraction.

Non-specific binding can be a problem in binding assays and attempts should be made to reduce the binding as much as possible. Three main factors are responsible for non-specific binding :-

1) Non-specific binding to sites in the tissue;

2) Free radiolabelled ligand that is not washed out of the tissue;

3) Binding of the ligand to some other component in the assay such as filters, tubes etc.

This third point has in effect been dealt with by the use of PEI treated filters. However, it is worth noting here that this problem was also encountered with the assay of (^{125}I) α bungarotoxin binding to the P2H fraction and this was overcome by allowing the eppendorf tubes to stand for 1 hour containing a solution of 1% BSA in PBS followed by two washes of PBS, before the binding assay was set up. The problem of unbound radioligand that has not been washed out of the tissue can obviously be overcome by an increase in the washing process, but this in turn may cause greater dissociation of the receptor ligand complex, thereby decreasing the signal. A balance must therefore be struck between signal and non-specific backgound. The most common way to determine binding to non-specific sites in the tissue is to use a pharmacologically related displacing ligand. However, when studying a binding site in a tissue which has not previously been characterised, there is no way of knowing what is the best related ligand to use and therefore a better approach is to first determine the kinetics of binding with non-specific binding determined by unlabelled ligand followed by a pharmacological study of the binding site. This is the approach that has been adopted in this study with unlabelled (-)nicotine used to determine non-specific binding for (^{3}H)(-)nicotine and unlabelled αbungarotoxin used to determine the non-specific binding for (^{125}I) α bungarotoxin.

Specific binding to the locust ganglionic P2 fraction was detected for both (^{3}H)(-)nicotine and (^{125}I) α bungarotoxin and was shown to increase linearly with increasing protein concentration thereby satisfying one of the criteria for identification of neurotransmitter receptors. (^{125}I) α Bungarotoxin binding was shown to saturate well below 10 nM but this was not the case for (^{3}H)(-)nicotine binding which did not fully saturate even at a concentration of 100 nM. However, analysis of the (^{3}H)(-)nicotine binding data suggested that the binding would saturate at higher concentrations although this must be interpreted with caution since Scatchard analysis of binding data depends on using a concentration range giving 10-90% occupancy of the binding sites. The results calculated for the Kd and Bmax for (^{3}H)(-)nicotine and (^{125}I) α bungarotoxin binding to the locust ganglionic P2 fraction were compared with values for rat brain P2 membranes obtained in a parallel study (MacAllan et. al., 1988) and these are shown in table 14. The results obtained for (^{125}I) α bungarotoxin binding to the locust ganglionic P2 fraction are in good agreement with the previously published Kd and Bmax values for other insects (see table 1 and Sattelle, 1986). Moreover, the Kd for (^{125}I) α bungarotoxin binding to the locust ganglionic P2 fraction was very similar to that obtained for the rat brain P2 membranes. However, a large difference was observed in the calculated Kd for (^{3}H)(-)nicotine binding

to the two tissues, with the locust ganglionic P2 fraction exhibiting a much lower affinity, although it is important to remember that this value was calculated from a concentration range that was not fully saturating (see above). Bmax values for both radioligands binding to the locust ganglionic P2 fraction were an order of magnitude greater than those observed for the rat brain P2 membrane fraction. It is interesting to note that the Bmax values for (^3H)(-)nicotine binding were 2-4 times more than for (^{125}I) α bungarotoxin binding in each tissue.

In rat brain, an endogenous inhibitor of (^3H)(-)nicotine binding has been identified which has been shown to affect the Kd for the binding of this ligand (Sershen et. al., 1984; Perry et. al., 1986). The nature of this inhibitor has not yet been properly established. To check if a similar inhibitor is present in locust ganglia, the P2 fraction was washed with either buffer or water. However, neither treatment was found to increase the affinity of (^3H)(-) nicotine binding. It is therefore assumed that there is no endogenous inhibitor for the (^3H)(-)nicotine binding site in the locust ganglia or if there is, the washing procedure tried does not remove it. An endogenous inhibitor of (^{125}I) α bungarotoxin binding has however been identified in homogenates of Locusta migratoria ganglia (Breer, unpublished). This inhibitor can be removed by washing the membrane homogenate which results

in an increase in (^{125}I) α bungarotoxin binding. Furthermore, the inhibitor has been purified and when added back to the washed membrane preparation inhibits (^{125}I) α bungarotoxin binding. In this study, binding experiments with (^{125}I) α bungarotoxin were performed on a membrane fraction that had been centrifuged and resuspended in fresh buffer and therefore an endogenous inhibitor of (^{125}I) α bungarotoxin binding may also be present in this tissue but it is likely that it would be washed out by the membrane fractionation procedure. The effect on the binding of further washing of the membrane fraction was not tested here.

Having identified differences in the binding sites for (^{3}H)(-)nicotine and (^{125}I) α bungarotoxin in locust ganglia, it was necessary to further characterise these sites by determining IC_{50} values for the inhibition of each radioligand by a range of other cholinergic ligands. However, IC_{50} values are dependent on the concentration of radioligand used in the assay. It is therefore important to work at a radioligand concentration close to the Kd to get as good an estimate as possible of the IC_{50} for the displacing ligand. In practice this is not always possible and in this study a concentration of 50 nM (^{3}H) (-)nicotine was used (cost of the radioligand precluded the use of higher concentrations) which is less than half the Kd of 130 nM. In contrast, the concentration of

(^{125}I) α bungarotoxin used for the inhibition studies was 1 nM compared with a Kd of 0.8 nM. It is, however, possible to make a direct comparison of these inhibition studies by eliminating the effect of the concentration of radioligand by converting the IC_{50} values to inhibition constants (Ki) using the formula

$$Ki = IC_{50}/(1 + L/Kd)$$

where L is the radioligand concentration and Kd is the dissociation constant for that radioligand (Cheng and Prussoff, 1973). Therefore Ki values have been used instead of IC_{50} values in this study.

Another requirement to be considered in inhibition studies is that the receptor binding site concentration should be less than 10% of the Kd for the radioligand (Jacobs et. al., 1975). In this study, the average receptor site concentration for (^{3}H)(-)nicotine binding in the inhibition studies was 1 nM and was therefore well below 10% of the Kd for (^{3}H)(-)nicotine binding. However, to get a reasonable signal for (^{125}I) α bungarotoxin binding, a receptor concentration of 0.32 nM was used which may result in the Ki values deviating slightly from the true value. It is very important when comparing inhibition results from different studies to take these factors of radioligand concentration and receptor site concentration into account.

The pharmacology of the binding of (^{3}H)(-)nicotine and (^{125}I) α bungarotoxin to the locust ganglionic P2 membrane fraction was studied using several cholinergic ligands, and in particular, two novel toxins, MLA and (+)anatoxin-a (the naturally occuring enantiomer). MLA has previously been shown to inhibit (^{3}H) α bungarotoxin binding to a membrane fraction of housefly heads with a Ki of 2.5 x 10^{-10}M and it is known to be a potent insecticide (Jennings et. al., 1986). (+)Anatoxin-a is a potent agonist of the vertebrate muscle nAChR (Spivak et. al., 1983 ; Spivak et. al., 1983) closely resembling channel properties evoked by ACh (Spivak et. al., 1983). This toxin has been shown to have a much lower affinity for the muscarinic binding site of rat brain (Aronstam and Witkop 1981). (+)Anatoxin-a and MLA were found to be equally potent inhibitors of (^{125}I) α bungarotoxin binding to the locust ganglionic P2 fraction. The classical nicotinic antagonist DHβE was found to be slightly more effective at this site, than both of these toxins, whereas, (-)nicotine was slightly less effective. Neither (+)nicotine or (-) anatoxin-a were very effective inhibitors of (^{125}I) α bungarotoxin binding to this tissue. Only (-) and (+) nicotine were found to effectively inhibit the binding of (^{3}H)(-)nicotine to the locust ganglionic P2 fraction, although the stereospecificity for the (-)enantiomer was low compared to that for the inhibition of (^{125}I)

α bungarotoxin. In contrast, the stereospecificity for (-) nicotine inhibition of the two radiolabelled ligands in rat brain P2 membranes was found to be greater against (^{3}H)(-)nicotine binding than (^{125}I) α bungarotoxin binding (Wonnacott 1986). All of the other cholinergic ligands tested for their ability to inhibit (^{3}H)(-)nicotine binding to the locust ganglionic P2 fraction were found to be virtually ineffective.

The rank order of potency of cholinergic ligands inhibiting (^{3}H)(-)nicotine and (^{125}I) α bungarotoxin binding to the locust ganglionic P2 fraction and rat brain P2 membranes is shown in table 15. It is clear that the order of potency for the inhibition of (^{125}I) α bungarotoxin binding in these two tissues is essentially similar with the exception of DHβE, which is the most effective inhibitor in locust ganglia but is only moderately effective in rat brain. The order is also very similar for the inhibition of (^{3}H)(-)nicotine and (^{125}I) α bungarotoxin binding in rat brain with the exception of MLA, which is the most potent at the (^{125}I) α bungarotoxin binding site but is only moderately effective at the (^{3}H)(-)nicotine binding site. MLA is obviously a good probe for neuronal (^{125}I) α bungarotoxin binding sites since it is not an effective inhibitor of (^{125}I) α bungarotoxin binding to the Torpedo nAChR (MacAllan et. al., 1988). Moreover, MLA shows a specificity only previously seen with the snake venom αtoxins.

The (^{125}I) α bungarotoxin binding site in locust ganglia certainly satisfies the third criterion for the identification of a neurotransmitter receptor whereas the (^{3}H)(-)nicotine binding site displayed no significant cholinergic pharmacology. Therefore, from these binding studies, it is concluded that in locust ganglia there is an (^{125}I) α bungarotoxin binding site which shares pharmacological specificity with both vertebrate neuronal and peripheral nAChR's, and there is also a specific (^{3}H)(-)nicotine binding site, the pharmacology of which appears to be non-cholinergic at this stage. However, it is interesting to note that early studies on the binding of (^{3}H)(-)nicotine to vertebrate brain tissue suggested that there were (^{3}H)(-)nicotine binding sites which were non-cholinergic based on the inability of nicotinic and muscarinic ligands to inhibit the binding (Abood et. al., 1980; Sershen et. al., 1981). More detailed studies on the binding of (^{3}H)(-)nicotine to vertebrate brain membrane preparations revealed that there are cholinergic (^{3}H)(-)nicotine binding sites present showing strong sensitivity to many nicotinic ligands and in particular those that are agonists (see Wonnacott, 1987 for review).

A previous study on (^{3}H)(-)nicotine binding to a membrane fraction from housefly heads and the nerve cord of the American and Madagascar cockroach suggested the presence of high affinity nicotine binding sites in these tissues

with Kd's of 3, 1.1 and 1.5 μM respectively (Aziz and Eldefrawi, 1973). These binding sites had low affinity for ACh, determined by inhibition studies, but exhibited both nicotinic and muscarinic pharmacology. The authors therefore concluded that this (^{3}H)nicotine binding site in insect membrane tissue was the same as the (^{3}H)decamethonium binding site identified in soluble extracts of insect nervous tissue (i.e. the mixed affinity site : see introduction section 1.11.1).

In a further attempt to characterise the identity of the (^{3}H)(-)nicotine binding site, in the locust ganglionic P2 fraction, as a neuronal nAChR, (^{3}H)MCC was tested for its ability to bind to these sites. (^{3}H)MCC has been shown to bind to high affinity binding sites in rat brain which can be potently inhibited by nicotinic ligands but only poorly inhibited by muscarinic ligands (Boksa and Quirion, 1987; Abood and Grassi, 1986). Moreover, the Kd and Bmax for this binding were found to be very similar to those for (^{3}H)(-)nicotine binding to rat brain tissue and it is generally accepted that they both bind to the same site. In this study, although the binding of (^{3}H)MCC to the locust ganglionic P2 fraction could be shown to increase with increasing concentration of the ligand, this binding could not be displaced by nicotine or by any other cholinergic ligand tested and the binding was therefore determined to be non-specific. However, (^{3}H)MCC would be

expected to bind to the (^{125}I) αbungarotoxin binding site since (-)nicotine is a good inhibitor of (^{125}I) α bungarotoxin binding. No inhibition of (^{3}H)MCC binding by α bungarotoxin could be detected and this may be explained if higher concentrations of (^{3}H)MCC are needed to get detectable binding to this site. Indeed at relatively high concentrations, of (^{3}H)MCC (75 nM and 100 nM), small levels of specific binding could be determined with (-)nicotine as the displacing ligand and this specific binding may represent binding of (^{3}H)MCC to the αbungarotoxin binding site. It would be interesting to investigate the effects of unlabelled MCC on the binding of (^{125}I) α bungarotoxin binding and calculate a Ki value for it, although unlabelled MCC is not available at present.

Therefore, the nature and specificity of the high affinity (^{3}H)(-)nicotine binding site in locust ganglia is still unclear and further work on this site will be necessary to demonstrate its significance and whether it represents part of a true nAChR. However, at present, according to the criteria for identification of neurotransmitter receptors, it remains just a specific binding site.

4.3 Purification and Analysis of the Locust αBungarotoxin Binding Protein

Having identified pharmacologically significant binding sites for a ligand, the next step is to purify the receptor protein. This has been attempted by several groups for the insect α bungarotoxin binding site (see introduction 1.11.4) but there is still controversy over the size and numbers of receptor subunits. Therefore further purification and analysis of the α bungarotoxin binding protein is required.

In this study, an initial analysis of the level of (^{125}I) α bungarotoxin binding sites present in the heads of the five instar stages was compared with that for the adult head, in an attempt to identify a stage which was particularly rich in this binding site. It was clear from the results that although the level of binding per head increased with each stage of development, the level of binding per mg of protein was relatively constant throughout development. The choice of material for the purification of the α bungarotoxin binding protein was therefore the adult insect ganglion.

Previous work in this laboratory (unpublished) suggested that the detergent Lubrol PX was the best detergent to use for the solubilisation of the locust ganglia αbungarotoxin binding protein. This detergent is a mild non-ionic

detergent which was used in the isolation of the chick optic lobe α bungarotoxin binding protein (Conti-Tronconi et. al., 1985). In the case of locust ganglia, maximum (^{125}I) α bungarotoxin binding activity was solubilised from the membrane with a Lubrol PX concentration of 3% (w/v). It is interesting to note here that (^{3}H)(-) nicotine did not bind to this detergent extract, and this might be explained by the (^{3}H)(-)nicotine binding site being very labile when extracted from the membrane, as has been noticed with the rat brain high affinity nicotine site (Wonnacott, personal communication).

Purification of the vertebrate peripheral α bungarotoxin binding protein has been accomplished by the use of affinity columns with immobilised Naja Naja toxin rather than α bungarotoxin, since the binding to Naja Naja toxin is more reversible than to α bungarotoxin for these vertebrate receptors. However, α bungarotoxin affinity columns have proved useful for the isolation of neuronal α bungarotoxin binding proteins and in particular for the insect α bungarotoxin binding protein since the affinity of these sites is much lower than that of the Torpedo or vertebrate muscle nAChR. In this study a method of recirculation of benzoquinonium through the affinity column and a DE52 ion exchange column was used to elute the α bungarotoxin binding protein bound to the affinity column. This method circumvented the need to dialyse away

the eluting ligand. Benzoquinonium was chosen as the eluting ligand because it is coloured red and is therefore easy to observe when applying it to the recirculation system and when washing it out. It is also a good inhibitor of (^{125}I) α bungarotoxin binding in locust ganglia (Filbin et. al., 1983). The quantity of protein purified from 100 ganglia was very small and certainly below the detection limits of any of the well known protein assay methods. However, an ultrasensitive protein assay at the nanogram level using colloidal gold has recently been reported (Stoscheck, 1987) which may prove useful in future studies of this protein. Estimates of the amount of protein purified were made from the results of (^{125}I) α bungarotoxin binding and were around 1 μg per 100 ganglia.

Enough protein was purified from about 25 locust ganglia to analyse by SDS-PAGE using a silver staining procedure. In these experiments, a predominant band with a Mr of 49,000 was found to be consistently present in all the preparations. How then does this protein relate to the α bungarotoxin binding protein purified from other insects? A similar protein has been purified from another species of locust, Locusta migratoria, and from the cockroach Periplaneta americana, both of which appear to be composed of one type of subunit with a Mr of 65,000 (see Breer and Sattelle, 1987). This single polypeptide

has been proposed to form a homo-oligomer structure (Breer et. al., 1985). It is clear from sequence analysis that the four genes coding for the subunits of the Torpedo nAChR have evolved from two and initially one ancestral gene (see introduction section 1.8.1, also see Raftery et. al., 1980) and it has been proposed that the locust nAChR single Mr 65,000 polypeptide may be coded for by the ancestral gene (Breer et. al., 1985). In this study on the Schistocerca gregaria α bungarotoxin binding protein, several of the purified preparations were observed to contain an additional band at Mr 66,000. In these preparations, it is unclear whether this additional band is part of the α bungarotoxin binding protein or whether it is another protein copurifying on the affinity column. The α bungarotoxin binding protein from Locusta migratoria was also purified in this study, and was found to have a single subunit of Mr 51,000. Another insect α bungarotoxin binding protein has been purified from housefly heads and has been shown to consist of two types of subunits with Mr's 42,000 and 25,000 (March et. al., 1982). The smaller of these two bands is thought possibly to be due to proteolysis.

A possible reason for the differences between these results from different groups is that a different detergent is used in each study. In this study Lubrol PX was chosen as detergent whereas Triton X-100 was used in

the case of housefly heads (March et. al., 1982) and sodium deoxycholate was used for Locusta migratoria (Breer et. al., 1985). In the case of the chick optic lobe α bungarotoxin binding protein, the detergent Lubrol PX was found to solubilise a component with a sedimentation coefficient of 9.1S compared to 12S for the related component from other brain regions (Norman et. al., 1982).

However, when Triton X-100 was used in place of Lubrol PX the sedimentation coefficient changed to the 12S value. These differences were attributed to different protein oligomers being formed in the initial extraction procedure rather than to the size of the receptor/detergent miscelles. However, SDS-PAGE analysis of these two preparations yielded essentially similar results with the major polypeptide at Mr 54,000 and a minor band at Mr 51,000, but in the triton X-100 purified samples an additional band at Mr 47,000 was present which disappeared with progressive purification, without affecting receptor activity, and was concluded not to be an integral part of the nAChR. Therefore it is unlikely that the use of different detergents in these insect preparations is the cause of different Mr results on SDS-PAGE and a more plausible reason for these differences could be due to the presence of proteolytic activity during the purification procedure, a situation observed in the early attempts to purify vertebrate nAChR's (Conti-Tronconi and Raftery,

1982). The Torpedo nAChR is now known to be a pentamer of four different types of subunit, although there was considerable controversy over the subunit composition in the early days of study (Conti-Tronconi and Raftery, 1982). It is now clear that proteolysis has a significant effect on the subunit pattern of the Torpedo receptor with the α subunit being more resistant to proteolysis than the β , γ or δ subunits, and it has been shown that inclusion of potent protease inhibitors allows the isolation of the receptor protein with all four intact subunits (Conti-Tronconi and Raftery, 1982). A similar situation was encountered in the attempts to purify the chick optic lobe α bungarotoxin binding protein (Conti-Tronconi et. al., 1985 ; Norman et. al., 1982). Furthermore, it has been demonstrated that proteolysis does not necessarily mean that the physical and functional properties of the receptor protein are affected since extensive proteolysis of the receptor protein by papain or trypsin does not significantly alter the binding capacity for α bungarotoxin, cholinergic ligands or antibodies, only slightly affects the sedimentation behaviour and the receptor protein can still be functionally reconstituted into lipid bilayer membranes (Conti-Tronconi and Raftery, 1982). However, only when the protein is denatured in SDS and subjected to electrophoresis does the damage become evident. Therefore, it is clear that the proteolytic damage can be in the form of nicks in the polypeptide

chain and the protein still holds together with a minimum degree of fragmentation.

With this in mind, it is possible that the polypeptide of Mr 49,000 purified from the locust ganglia in this study is a proteolysis product of the same protein purified by Breer. Similarly, the polypeptide of Mr 42,000 purified from housefly heads may also represent a proteolysis product of some larger protein and it is interesting to note here, that long storage of the Schistocerca gregaria α bungarotoxin binding protein at $4^{o}C$, in this study, resulted in a change in Mr of the predominant band from 49,000 to 43,000. To try to determine whether proteolysis was the cause of the difference in Mr between the Schistocerca gregaria and Locusta migratoria α bungarotoxin binding protein, several protease inhibitors used in the purification of vertebrate receptor proteins were included in the purification buffers in this study. However, no indication of protease inhibition was detected since the Mr of the predominant band remained at 49,000. The nature and specificity of proteases in the insect nervous system has been an area of little if any research and therefore there is no reason to assume that protease inhibitors active against vertebrate proteases will also be active against insect proteases.

Having purified the insect α bungarotoxin binding protein, the question then arises of whether this protein represents a functional nAChR. This has in fact been partly answered by the reconstitution of the αbungarotoxin binding protein from Locusta migratoria into artificial lipid bilayers, resulting in the formation of functional nAChR channels which were shown to respond to the cholinergic agonists carbamylcholine and suberyldicholine by a marked increase of conductance and this could be blocked by the nicotinic antagonist d-tubocurarine (Hanke and Breer, 1986). Moreover, single channel recording in physiological saline gave a conductance of 70-80 pS with a channel lifetime of a few millseconds. The Mr 65,000 polypeptide of the Locusta migratoria α bungarotoxin binding protein has been eluted from denaturing gels and reconstituted into artificial lipid bi-layers resulting in functional nAChR channels (Breer, 1988) and therefore it would seem like this polypeptide alone can form a functional nAChR channel. However, the effect of α bungarotoxin as an antagonist in these experiments was not reported. The rat brain α4 clone when expressed in oocytes directs the synthesis of a polypeptide chain which confers weak nicotinic responses to the oocyte membrane (Boulter et. al., 1987) and therefore it may be that nicotinic responses can be produced by a single type of subunit (i.e. the ligand binding subunit).

Therefore, all the criteria for the identification of the insect αbungarotoxin binding protein as a functional neurotransmitter receptor have been satisfied, but it is clear that controversy still surrounds the true molecular structure of this nAChR.

It seems likely that, as in the case of vertebrate brain, there may be heterogeneity of nAChR's in insect CNS. This hypothesis has recently been supported by the identification of presynaptic or extrasynaptic nAChR's in the membrane of a cholinergic axon in the cockroach first instar (Blagburn and Sattelle, 1987). The sensitivity of this axon of the lateral filiform hair sensory neurone to iontophoretically applied carbamylcholine was found to be similar to that of the dendritic regions of giant interneurone 3 (known to contain postsynaptic nAChR's). Nicotine was shown to mimick the carbamylcholine responses in these two neurones and both the nicotine and carbamylcholine evoked response in both neurones were blocked by 10^{-5}M mecamylamine and 10^{-6}M d-tubocurarine but not by muscarinic antagonists. It has been suggested that the nAChR's on the presynaptic axon may represent nicotinic autoreceptors although direct evidence to support this has not yet been obtained.

4.4 Affinity Labelling of the Locust nAChR

Affinity labelling of neurotransmitter receptors is a useful technique to identify the ligand binding subunit and the actual location of the ligand binding site within that subunit. There are two types of affinity labelling regularly used in the study of neurotransmitter receptors, namely photoaffinity labelling and affinity labelling with alkylating compounds. Photoaffinity labelling requires the use of a photoactivatable ligand for the receptor, such as flunitrazepam, which has been used for the study of the benzodiazepine binding site of the locust ganglia GABA-receptor complex (Robinson et. al., 1986). The other type of affinity labelling has been used for the study of the nAChR and involves the compound (^{3}H)MBTA which has an affinity for the cholinergic binding site and also contains a sulphydryl alkylating group which will alkylate reduced cysteine residues. In this way the αsubunit of the Torpedo and vertebrate muscle nAChR have been identified as the ligand binding subunit and the cysteine residues at positions 192 and 193 (Torpedo αsubunit) have been located at the ligand binding site of the protein (Kao et. al. 1984). A previous report, from this laboratory on the αbungarotoxin binding protein in Schistocerca gregaria indicated that (^{3}H)MBTA labelled a polypeptide of Mr 58,000 in a P2

membrane fraction (Filbin et al., 1983) whereas a report on the housefly head αbungarotoxin binding protein demonstrated that (^{3}H)MBTA labelled a polypeptide of 42,000 (March et al., 1982). In this study, a polypeptide of Mr 49,000 in the P2 membrane fraction was labelled with (^{3}H)MBTA. This (^{3}H)MBTA labelled polypeptide corresponds exactly to the Mr of the purified αbungarotoxin binding protein from locust ganglia in this report and it is likely that they are the same protein. The difference between the Mr for the labelled protein in this study and that reported by Filbin et. al. (1983) may be attributable to differences in SDS-PAGE techniques. How then, do these affinity labelling results for insects compare to studies on vertebrate brain? (^{3}H)MBTA has been shown to label a subunit of an αbungarotoxin insensitive nAChR in chick brain with a Mr of 59,000 (Whiting and Lindstrom, 1987a). However, in the study of the vertebrate brain α bungarotoxin binding protein, BACh was used as the affinity ligand in place of (^{3}H)MBTA. In chick brain, a subunit polypeptide of Mr 56,000 was labelled with this ligand (Conti-Tronconi et. al., 1985) and this corresponds well with the αbungarotoxin binding protein from rat brain in which the Mr 55,000 subunit was labelled (Kemp et. al., 1985).

4.5 Cross-Reactivity of the Locust αBungarotoxin Binding Protein with Other nAChR's

A commonly used approach to the study of nAChR's is to look for crossreactivity between receptors from different species. An initial study with an anti Torpedo nAChR antiserum demonstrated that there was no crossreactivity between the Torpedo nAChR and the detergent solubilised α bungarotoxin binding protein from housefly heads (Eldefrawi and Eldefrawi, 1980). More recently however, Mab's against the Torpedo nAChR have shown that there is crossreactivity between this receptor and the putative nAChR in insects (see introduction section 1.11.6). In the present study, an antiserum against the purified Torpedo nAChR was found to precipitate a small amount of (^{125}I) αbungarotoxin binding activity from the detergent extract suggesting a low level of crossreactivity. It is not surprising that only low crossreactivity was detected with this antiserum since it is known that the crossreactivity between the Torpedo and vertebrate muscle nAChR can be low (Lindstrom et. al., 1979). Mab's raised against the Torpedo nAChR and vertebrate brain high affinity nicotine sites were unable to precipitate (^{125}I) α bungarotoxin binding activity from the detergent extract in this study. However, since Mab's are directed against only one epitope of a protein it is less common to find crossreactivity with a Mab than with an antiserum and many more Mab's would have to be tested to find one that does crossreact.

4.6 The application of Molecular Biology to the Study of the Insect nAChR

It is becoming increasingly clear that the techniques of molecular biology are a valuable asset to the neurochemist for the study of neurotransmitter receptors and use of these techniques has been exploited for the study of the Torpedo and vertebrate muscle nAChR's and more recently for the study of vertebrate neuronal nAChR's (see introduction sections 1.8, 1.9 and 1.10.5). Molecular biology methodology is now also being applied to the study of the insect nAChR (see introduction section 1.11.8).

Before the genes encoding the receptor protein can be isolated, a suitable probe is required and in the case of the Torpedo nAChR this was obtained by sequencing the N terminus of each of the receptor subunits. An oligonucleotide probe was then constructed corresponding to the N terminal sequence and used to probe for the corresponding gene. Therefore, attempts were made in this present study to obtain N terminal sequence for the purified α bungarotoxin binding protein. However, for each attempt to get sequence data at least one thousand ganglia are required as starting material and therefore the cost of several attempts was prohibitive. The one attempt that was made resulted in the protein having a blocked N terminal and no further attempts were made.

In collaboration with John Marshall at the MRC Molecular Neurobiology Unit, Cambridge, initial attempts were made to express the locust ganglia α bungarotoxin binding protein by injecting poly A^+ mRNA from locust ganglia into the Xenopus oocyte expression system. Significant levels of (^{125}I) α bungarotoxin binding sites were detected in detergent extracts of these injected oocytes, comparable to oocytes that had been injected with Torpedo poly A^+ mRNA. Therefore it was concluded that the locust ganglia was a good source of receptor specific mRNA, from which a cDNA library could be constructed and used to isolate clones coding for the locust ganglia nAChR. Initial results of this cloning work have now been reported by Marshall et. al., (1988a). During this early work on the expression in oocytes, the group of Breer also reported the expression of (^{125}I) α bungarotoxin binding sites in oocytes, directed by poly A^+ mRNA from Locusta migratoria ganglia (Breer and Benke, 1985). This was followed by a publication reporting the immunoprecipitation of polypeptides, synthesised from the poly A^+ mRNA in oocytes, with a monospecific antiserum to the Locusta migratoria purified α bungarotoxin binding protein. The only precipitated polypeptide was shown to have a Mr of 65,000 corresponding to the purified α bungarotoxin binding protein (Breer and Benke, 1986).

As mentioned above, cDNA clones have now been isolated which are thought to code for subunits of the insect nAChR (see introduction section 1.11.8). In the case of the locust, Schistocerca gregaria, a genomic clone was initially isolated using a chick brain nAChR cDNA probe (Marshall et. al., 1988a) and a 400 base pair fragment of this clone was subsequently used to screen a λgt 10 cDNA library from locust (Marshall et. al., 1988b) resulting in the isolation of two non-identical cDNA clones named ARL1 and ARL2. One of these clones has been proposed to correspond to an alpha-like polypeptide and the other a non-alpha polypeptide of a nAChR. Part of the sequence of the non-alpha clone corresponds to a highly conserved region found in all nAChR subunits and also in other ion channel receptors (see introduction section 1.12). This sequence is identical to a sequence found in the fruitfly non-alpha ARD clone (Hermans-Borgmeyer et. al., 1986) and is able to form a loop structure linked by the two cysteine residues (see introduction section 1.12). Both the ARD clone and locust non-α clone show considerable homology over this region to another isolated Drosophila clone, the ALS clone which is thought to be an α-like subunit of the insect nAChR (see introduction section 1.12 and figure 8).

In this study, a peptide corresponding to this cysteine loop region was synthesised with modified cysteine

residues so that the synthesised peptide would not be disulphide linked. However, it is likely that the peptide would adopt a conformation that is similar to the disulphide linked form since a β hairpin loop is induced by the proline residue whether the two cysteines are linked or not. It was assumed that this unlinked form of the peptide would still be able to elicit an antibody response which would recognise the native insect nAChR since a recent report has shown that a non-disulphide linked peptide corresponding to the sequence 125-147 (encompassing the cysteine loop region) of the human muscle α subunit was able to generate an antiserum with myasthenic activity (McCormack et. al., 1987) implying crossreactivity with the native receptor.

The antiserum raised against the synthetic peptide in this study was not able to precipitate (^{125}I) α bungarotoxin binding activity from the detergent extract of the locust ganglionic P2 fraction. It did however identify two specific bands in Western blots of SDS-PAGE using the locust ganglionic P2 fraction as antigen. These bands correspond to Mr's of 49,000 and 60,000 with the 49,000 band being the strongest. This antiserum was also shown to react with the purified αbungarotoxin binding protein in a Western blot. The polypeptide of Mr 49,000 which binds α bungarotoxin and (^{3}H)MBTA is by analogy to the vertebrate receptors an α-like subunit. However, the

peptide sequence was derived from a proposed non-α subunit and may therefore indicate a high degree of homology between these two identified polypeptides over this cysteine loop region. The faint band at Mr 60,000 observed in the Western blot of the P2 fraction may be the protein encoded by the non-α clone and its low abundance compared to the band at Mr 49,000 may be due to effect of proteolysis as discussed earlier (section 4.3). Alternatively, the band at Mr 60,000 may be the same protein as the Mr 49,000 band which has not been proteolysed to the same extent or may represent a subunit of a different ion channel receptor. It is likely that antibodies to this synthetic peptide could cross react with polypeptide chains of other ion channel receptors since it is becoming clear that all ion channel receptor proteins belong to a super family of proteins with considerable sequence homology in their polypeptides particularly in the cys loop region (see introduction section 1.12).

A final piece of evidence to back up the proposal that the non-α clone encodes a subunit of the locust nAChR came from antibody studies. An antiserum that had been raised to the purified α bungarotoxin binding protein from Schistocerca gregaria was shown to recognise the synthetic peptide indicating crossreactivity of the peptide with the α bungarotoxin binding component.

More work on these ARL1 and ARL2 clones is currently underway and the full coding sequences will soon be known. Recently, it has been reported that these two clones have been injected into Xenopus oocytes resulting in the appearance of electrophysiological responses to bath-applied nicotine (Marshall et. al., 1988b).

4.7 Future Research on the Insect nAChR

This thesis has reported improved characterisation of the locust nAChR. There is still a large amount of work to be tackled in this field however and future research is likely to be concentrated more towards the use of molecular biology to characterise the receptor. However, it is essential to back up the results obtained by molecular biology with those from biochemical and physiological studies.

Initial studies show extensive homology of the insect receptor with the vertebrate receptors and in particular the vertebrate neuronal nAChR's. It is possible that insect ganglia may also contain heterogeneous populations of nAChR's and therefore more detailed comparisons of vertebrate and invertebrate receptors are required. This may be of interest to the insecticide companies if differences are identified which could be used in rational design programmes for the synthesis of new insecticides.

Computer modelling is likely to make a major impact on our knowledge of both the vertebrate and insect nAChR's and once the full tertiary structure for the Torpedo nAChR is known, it should not be long before computer generated three dimentional models of the other nAChR's, including the insect nAChR, will be available.

CONCLUDING COMMENTS

(1) This study has made use of α-bungarotoxin as a probe of the nAChR. What evidence is there to suggest that the α-bungarotoxin binding protein in locust ganglia is a functional nAChR? There is no direct evidence for the locust Schistocerca gregaria and therefore a comparison has had to be made with other insect species. An α-bungarotoxin binding protein purified from another species of locust, Locusta migratoria, has been reconstituted into planar lipid bilayer membranes (Hanke and Breer, 1986). Functional ion channels have been identified in these membranes which respond to suberyl dicholine and carbamyl choline. However, there was no report on whether or not α-bungarotoxin could block these agonist evoked responses. Electrophysiological studies on the cockroach CNS have demonstrated that α-bungarotoxin can block synaptic transmission (Sattelle et. al., 1983) and nAChR responses to iontophoretically applied nicotinic agonists (Harrow and Sattelle, 1983).

(2) SDS-PAGE analysis of the α-bungarotoxin binding protein, purified from Schistocerca gregaria ganglia, demonstrated that a polypeptide of Mr 49,000 was consistently present in different samples. However, in three of the samples an additional polypeptide of Mr 66,000 was also present. A previous study on the Schistocerca gregaria α-bungarotoxin binding protein suggested the presence of polypeptides with Mr 60,000, 41,000 and 25,000 (Filbin et. al., 1983) but it is not clear how these polypeptides relate to the results in this study. The α-bungarotoxin binding protein from Locusta migratoria, has been shown to consist of a predominant polypeptide with Mr 65,000 (Breer et. al., 1985). It is not clear whether the Schistocerca gregaria Mr 49,000 polypeptide is a proteolytic product of the Mr 66,000 polypeptide that is occasionally present in these purified samples or if they are different subunits of the α-bungarotoxin binding protein. If proteolysis

is the reason, then the α-bungarotoxin binding protein from these two species of locust may be very similar. It has been proposed that the α-bungarotoxin binding protein from Locusta migratoria is a homo-oligomer of the Mr 65,000 polypeptide (Breer et. al., 1985). However, it is possible that the α-bungarotoxin binding protein from both of these species of locust contain other subunits in addition to the predominant polypeptide, and their absence on denaturing gels could be due to proteolysis. Putative nAChR α-like and non-α-like subunit genes have been isolated from locust, Schistocerca gregaria (Marshall et. al., 1988), and fruitfly, Drosophila melanogaster (Hermans-Borgmeyer et. al., 1986; Bossy et. al., 1988), thereby lending support to the idea that the α-bungarotoxin binding protein consists of more than one subunit type.

(3) Four antisera were raised to a synthetic peptide corresponding to the cysteine loop region from the non-α-like gene of Schistocerca gregaria (Marshall et. al., 1988). All four antisera were strongly reactive against the peptide but only one was found to have low crossreactivity with specific proteins of the ganglionic P2 fraction on Western blots (a strong band at Mr 49,000 and a weaker band at Mr 60,000). Very low crossreactivity of this antiserum with the purified α-bungarotoxin binding protein was also observed. The reason for the low crossreativity may be explained by differences in the conformation of the immunising peptide and the conformation of the proteins on the Western blots.

(4) A critical experiment in this study was the identification of a polypeptide of the ganglionic P2 fraction that was specifically labelled with [^{3}H]MBTA to a greater extent than any other. The Mr of this polypeptide (Mr 49,000) was the same as the Mr of the major polypeptide of the purified α-bungarotoxin binding protein and the stronger reacting of the two polypeptides identified with the antipeptide antiserum.

However, there was a high background labelling in these experiments and it is possible that other polypeptides were also specifically labelled. A previous study on Schistocerca gregaria suggested that a polypeptide of Mr 58,000 was labelled with [^{3}H]MBTA (Filbin et. al., 1983). There have been no reports of affinity labelling experiments for Locusta migratoria.

(5) [^{3}H](-)Nicotine would be expected to bind to the α-bungarotoxin binding protein in Schistocerca gregaria ganglia based on the calculated Ki value for the inhibition of [^{125}I]α-bungarotoxin binding by (-)nicotine. The [^{3}H](-)nicotine binding in this study was determined non-cholinergic suggesting that it was not binding to the same site as [^{125}I]α-bungarotoxin. However, it is possible that the [^{3}H](-)nicotine binding to this tissue preparation was largely non-specific and masking the true [^{3}H](-)nicotine binding to the [^{125}I]α-bungarotoxin binding site.

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Phosphate Buffers

TABLE B.10 Preparation of 0.1 M Potassium Phosphate Buffer at 25°C

pH	Volume of 1 M K_2HPO_4 (ml)	Volume of 1 M KH_2PO_4 (ml)
5.8	8.5	91.5
6.0	13.2	86.8
6.2	19.2	80.8
6.4	27.8	72.2
6.6	38.1	61.9
6.8	49.7	50.3
7.0	61.5	38.5
7.2	71.7	28.3
7.4	80.2	19.8
7.6	86.6	13.4
7.8	90.8	9.2
8.0	94.0	6.0

Data from Green (1933).

TABLE B.11 Preparation of 0.1 M Sodium Phosphate Buffer at 25°C

pH	Volume of 1 M Na_2HPO_4 (ml)	Volume of 1 M NaH_2PO_4 (ml)
5.8	7.9	92.1
6.0	12.0	88.0
6.2	17.8	82.2
6.4	25.5	74.5
6.6	35.2	64.8
6.8	46.3	53.7
7.0	57.7	42.3
7.2	68.4	31.6
7.4	77.4	22.6
7.6	84.5	15.5
7.8	89.6	10.4
8.0	93.2	6.8

Data from ISCO (1982).

Dilute the combined 1 M stock solution to 1000 ml with distilled H_2O. pH is calculated according to the Henderson-Hasselbalch equation:

$$\text{pH} = \text{pK}' + \log\left[\frac{(\text{proton acceptor})}{\text{proton donor}}\right]$$

where pK′ = 6.86 at 25°C.

Ann Clin Biochem 1981; **18**: 146–152

An assessment of radioimmunoassay procedures for determination of anti-acetylcholine receptor antibodies in the sera of patients with myasthenia gravis

B CARTER, R HARRISON, G G LUNT, H MORRIS, T SAVAGE-MARENGO, AND F A STEPHENSON

From the Department of Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY

SUMMARY A reproducible radioimmunoassay procedure for the determination of anti-acetylcholine receptor antibodies in the sera of patients with myasthenia gravis is described and examined in detail. The assay combines features of a number of methods previously outlined and allows repeat determinations of antibody titre in a given myasthenic serum sample with coefficient of variation 6%. The mean ± standard deviation for normal human serum anti-acetylcholine receptor antibodies was found by this procedure to be 0·024 ± 0·033 nmol/l α-bungarotoxin binding sites whereas the range for myasthenic patients was 0–139·14 nmol/l with a mean value of 7·55 nmol/l α-bungarotoxin binding sites.

Myasthenia gravis is generally recognised to be an autoimmune disease in which patients develop an immune response to self nicotinic acetylcholine receptor (AChR) at the neuromuscular junction.[1] Approximately 90% of myasthenia patients show elevated serum levels of anti-AChR antibodies,[2] and there is an increasing demand for determination of such levels as an aid to diagnosis. Despite the usual presence of anti-AChR antibodies in the sera of myasthenia gravis patients, correlation of antibody titre with severity of symptoms is generally poor between patients.[3–5] Nevertheless, titres followed for a given patient over a period of time can reflect the clinical state of the patient, and serial assays can be used to monitor the effects of particular treatment regimens.[6]

A basic radioimmunoassay for the determination of anti-AChR antibody titres in human sera has been reported by a number of groups of workers.[3 7–10] The assay involves specific labelling of human AChR with radiolabelled snake venom α-toxin followed by the use of the labelled AChR, in excess, to precipitate anti-AChR antibodies present in the patient's serum. The assay is relatively complex, and a number of aspects of the published procedures differ widely, depending upon the authors. In many cases, moreover, exact experimental details are unclear, and the reasons underlying the choice of a particular technique or set of conditions are unspecified. We now report an examination of a number of variable factors in the radioimmunoassay, the results of which lead to an overall method suitable for routine application.

Material and methods

Myasthenic and normal human sera were quickly frozen and stored at −20°C before assay. Human muscle was obtained from lower limb amputations. Within 15 minutes of amputation, calf muscle was crudely dissected free from fat, tendon, and skin, transported in ice or solid carbon dioxide (approx. 20 minutes), and stored at −80°C.

α-Bungarotoxin from *Bungarus multicinctus* was purchased from Boehringer, Mannheim, West Germany as a lyophilised powder (1 mg), which was dissolved in 0·05 mol/l potassium phosphate buffer, pH 7·5 (2 ml), and stored at −20°C before use. Na ^{125}I in dilute NaOH solution (100 mCi/ml) was supplied by the Radiochemical Centre, Amersham, UK, and benzoquinonium chloride was a generous gift from Stirling Winthrop Inc, Rensselaer, NY, USA. All other chemicals were from Sigma Chemical Co, Kingston upon Thames, UK, or from BDH Chemicals, Poole, Dorset, UK.

^{125}I-Labelled α-bungarotoxin was prepared

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^{125}I-Labelled α-bungarotoxin was pre

hours at 22°C). The resulting precipitate was separated by centrifugation at 3000 *g* for 10 minutes, and the pellet was washed twice with 0·01 mol/l potassium phosphate buffer, pH 7·5, containing 0·15 mol/l NaCl, 1% (v/v) Triton X–100, and 0·1% (w/v) NaN_3 and counted for radioactivity. Specific binding of α-toxin was blocked in parallel incubations performed as above but containing additionally 1·0 mmol/l benzoquinonium chloride (or 0·1 mmol/l d-tubocurarine or 0·1 mmol/l non-radioactive bungarotoxin). Subtraction of the counts so obtained gave specifically-bound radioactivity in the test sample, which can then be related to the concentration of α-bungarotoxin-binding sites in the AChR extract. Thus the antibody titre is expressed as moles of specific α-bungarotoxin binding sites precipitated per litre of serum.

Maximal formation of AChR-antibody complexes was routinely checked by repeating the assay using serial twofold dilutions (with normal human sera) of antisera in order to ensure a linear relation between the volume of undiluted serum and precipitated radioactivity.

Results and discussion

The concentration of soluble AChR in the primary detergent extracts of human muscle was found to vary from 0·1 to 2·5 nmol/l α-bungarotoxin-binding sites over 20 preparations with a mean value of 0·52 nmol/l toxin-binding sites. This represents a mean recovery of 0·49 pmol toxin-binding sites per gram muscle.

The wide variation in α-bungarotoxin-binding activities of different muscle samples may reflect differences in the clinical state of the muscle samples before amputation. Muscle was always obtained from legs amputated because of either vascular disorders or diabetic gangrene, and it is possible that the different samples had been subjected to varying periods of ischaemia with attendant partial autolysis. Moreover most amputees were patients over 60 years of age who are known[14] to be subject to significant and varying degrees of motor denervation. This raises the further possibility of variable extents of acetylcholine receptor proliferation[15] in the muscle samples we examined, although this was not reflected in binding studies carried out by us on purified receptor preparations.[16]

Attempts to prepare useful extracts of acetylcholine receptor from postmortem muscle proved to be generally unsatisfactory, giving receptor concentrations of less than 0·1 nmol/l toxin binding sites. This probably reflects the occurrence of autolysis during the extended periods (more than 6 hours) elapsing between death and freezing of the muscle.

With regard to the method of extraction of AChR, the inclusion in the buffers of phenylmethylsulphonyl fluoride and benzethonium chloride as anti-proteolytic agents was found generally to improve the yield of AChR. Similar yields of AChR were found whether extraction in Triton-containing buffer was carried out for 16 hours at 4°C or for 3 hours at 20°C.

The concentration of AChR extract (0·5 nmol/l) recommended in the Methods section for use in the radioimmunoassay of anti-AChR antibody titres is only slightly less than the mean value in the extracts. This inevitably means that many preparations contain concentrations of receptor suboptimal for this purpose, and re-extraction of the muscle homogenate (Methods section) was found to give secondary extracts with $46 \pm 33\%$ ($\pm$ SD, 6 preparations) of the toxin-binding activity of the primary extract. Such re-extraction was often worthwhile in the case of relatively high-activity receptor preparations in order to supplement stocks of useful receptor.

Storage of AChR extracts at 4°C for periods of up to three months led to less than 40% loss of toxin-binding activity.

In the determination of AChR concentration, pre-incubation of AChR with ^{125}I-labelled α-bungarotoxin for periods of longer than 15 minutes, up to 5 hours, was found not to affect the results. On the other hand, incubation of the AChR-toxin complex with $(NH_4)_2SO_4$ for less than 16 hours led to diminished precipitation of radioactivity.

Separation of AChR-toxin complex from free toxin can be effected alternatively by filtration through DEAE cellulose filter discs on a Millipore filtration system[10] followed by washing the discs with 0·01 mol/l potassium phosphate buffer, pH 7·5, containing 0·1% (w/v) bovine serum albumin, 0·01% (w/v) NaN_3, and 1·0% (v/v) Triton X-100. In our hands, specifically-bound radioactive counts were consistently found to be 60–85% lower than those obtained by using $(NH_4)_2SO_4$ precipitation, a decrease that is at least partially attributable to saturation of the filters by the high levels of protein in the extract.[9 17] These levels can be reduced by dilution of the extract before filtration, which can lead, however, to unacceptable loss of sensitivity in the case of lower activity extracts. The DEAE filter assay is, nevertheless, much faster than that based on $(NH_4)_2SO_4$ precipitation and can be used to give rapid estimates of AChR concentration for use in the radioimmunoassay of anti-AChR antibodies.

A third method of separation of AChR-toxin complexes from free toxin in the routine assay of AChR concentrations involves the addition of excess myasthenic serum followed by goat

hours at 22°C). The resulting precipitate was separated by centrifugation at 3000 *g* for 10 minutes, and the pellet was washed twice with 0·01 mol/l potassium phosphate buffer, pH 7·5, containing 0·15 mol/l NaCl, 1% (v/v) Triton X–100, and 0·1% (w/v) NaN_3 and counted for radioactivity. Specific binding of α-toxin was blocked in parallel incubations performed as above but containing additionally 1·0 mmol/l benzoquinonium chloride (or 0·1 mmol/l d-tubocurarine or 0·1 mmol/l non-radioactive bungarotoxin). Subtraction of the counts so obtained gave specifically-bound radioactivity in the test sample, which can then be related to the concentration of α-bungarotoxin-binding sites in the AChR extract. Thus the antibody titre is expressed as moles of specific α-bungarotoxin binding sites precipitated per litre of serum.

Maximal formation of AChR-antibody complexes was routinely checked by repeating the assay using serial twofold dilutions (with normal human sera) of antisera in order to ensure a linear relation between the volume of undiluted serum and precipitated radioactivity.

Results and discussion

The concentration of soluble AChR in the primary detergent extracts of human muscle was found to vary from 0·1 to 2·5 nmol/l α-bungarotoxin-binding sites over 20 preparations with a mean value of 0·52 nmol/l toxin-binding sites. This represents a mean recovery of 0·49 pmol toxin-binding sites per gram muscle.

The wide variation in α-bungarotoxin-binding activities of different muscle samples may reflect differences in the clinical state of the muscle samples before amputation. Muscle was always obtained from legs amputated because of either vascular disorders or diabetic gangrene, and it is possible that the different samples had been subjected to varying periods of ischaemia with attendant partial autolysis. Moreover most amputees were patients over 60 years of age who are known[14] to be subject to significant and varying degrees of motor denervation. This raises the further possibility of variable extents of acetylcholine receptor proliferation[15] in the muscle samples we examined, although this was not reflected in binding studies carried out by us on purified receptor preparations.[16]

Attempts to prepare useful extracts of acetylcholine receptor from postmortem muscle proved to be generally unsatisfactory, giving receptor concentrations of less than 0·1 nmol/l toxin binding sites. This probably reflects the occurrence of autolysis during the extended periods (more than 6 hours) elapsing between death and freezing of the muscle.

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alternatively by incubation for 1, 2, or 4 hours at 22°C.

As described in the Methods section, serial dilutions of myasthenic sera were made routinely in order to ensure that the antibody assay was being done with sufficient molar excess of labelled AChR. An examination of the dependence of apparent antibody titres on the molar ratio of labelled AChR to specific antibodies showed that molar ratios of approximately 3:1 were sufficient to ensure maximal precipitation of anti-AChR antibodies in the myasthenic samples tested (Fig. 2). Maximal formation of AChR-anti-AChR antibody complexes requires not only a minimum AChR-antibody ratio but also a minimum concentration of AChR, which in turn depends upon the dissociation constants of the AChR-antibody complexes. A study of three myasthenic sera showed (Fig. 3) that minimal concentrations of AChR of 0·3–0·5 nmol/l, depending on the serum sample, were sufficient to effect maximum formation of complex. This range is just below the level 0·5 nmol/l of AChR concentration suggested for use in the radioimmunoassay. Nevertheless the results indicate that the latter value may be close to a minimum acceptable figure for some myasthenic sera and that higher concentrations of receptor, if they can be obtained, may well be used to advantage in the radioimmunoassay procedure.

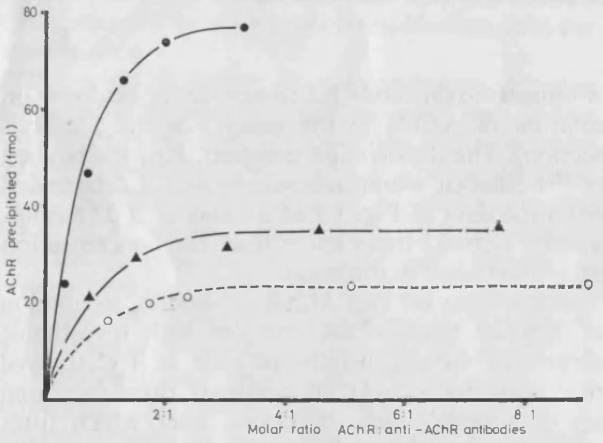

Fig. 2 *Formation of AChR-anti-AChR antibody complexes at varying molar ratios of toxin-labelled AChR to antibody. A single appropriate dilution of serum was made from each of three different myasthenic patients. Samples (5 μl) of each were incubated with increasing amounts of a fixed concentration of toxin-labelled AChR, and labelled AChR-antibody complexes were precipitated and counted as described for the antibody assay procedure (see text). The molar concentrations of antibody were, in each case, obtained from the maximum amount of AChR precipitated. Concentrations of toxin-labelled AChR were ●, 1·02 nmol/l, ▲, 1·32 nmol/l, and ○, 0·32 nmol/l.*

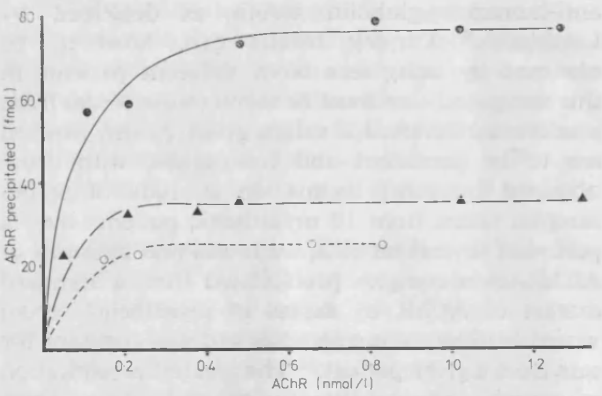

Fig. 3 *Formation of AChR-anti-AChR antibody complexes by varying concentrations of toxin-labelled AChR. A single appropriate dilution of serum was made from each of three different myasthenic patients. Samples (5 μl) of each were incubated with a constant amount of toxin-labelled AChR at different concentrations so that a constant molar ratio of toxin-labelled AChR: anti-AChR antibodies was maintained. These ratios were ●, 3:1; ▲, 7:1; ○, 10:1. Labelled AChR-antibody complexes were precipitated and counted as described for the antibody assay procedure (see text).*

The procedure for radioimmunoassay of anti-AChR antibodies outlined in the Methods section allows the determination of relatively reproducible titres. Thus, repeat determinations on an individual serum sample using a single extract of acetylcholine receptor and a single freshly prepared sample of ^{125}I-labelled α-bungarotoxin gave values of 2·99 ± 0·17 (± SD, 15 assays) nmol/l α-bungarotoxin binding sites. Repeat assays of the same serum using a single extract of acetylcholine receptor and four different preparations of ^{125}I-labelled α-bungarotoxin gave values of 3·23 ± 0·31 (± SD) nmol/l α-bungarotoxin binding sites (where the mean value for each toxin preparation resulted from 15 separate assays). Repeat assays of a different serum sample using a single preparation of ^{125}I-labelled α-bungarotoxin and four different extracts of acetylcholine receptor gave values of 1·80 ± 0·22 (± SD) nmol/l α-bungarotoxin binding sites (where the mean value for each extract of acetylcholine receptor resulted from 10 separate assays).

It can be seen that rather more variability in these assays is usually found when different ^{125}I-labelled α-bungarotoxin and acetylcholine receptor preparations are used. Titres obtained for a given serum sample using a single extract of acetylcholine receptor and sample of labelled toxin change little over a period of less than three weeks. Thus repeat assays of a single serum sample using one extract of acetylcholine receptor made using freshly ^{125}I-labelled α-bungarotoxin and the same toxin after

alternatively by incubation for 1, 2, or 4 hours at 22°C.

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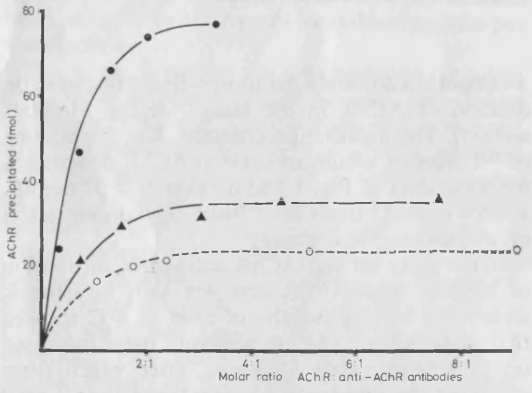

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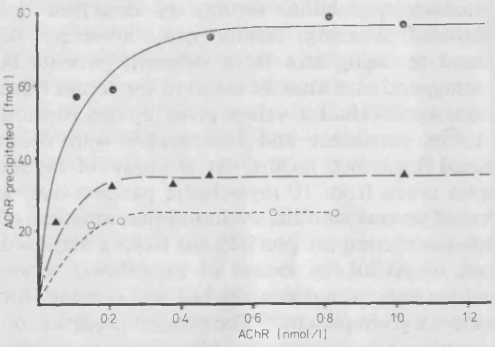

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Accepted for publication 29 January 1981